Neuropeptide Antagonists for Cancer Treatment

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List of Abbreviations

AFU: Arbitrary Fluorescent Units
Ann V: Annexin V
AO: Acridine Orange
APCIMS: Atmospheric Pressure Chemical Ionisation Mass Spectrometry
Arg: Arginine
AVP: Arginine Vasopressin
BBN: Bombesin
BK: Bradykinin
Boc: tert-Butyloxycarbonyl
CCK: Cholecystokinin
CDK: Cyclin Dependent Kinase
CDKI: Cyclin Dependent Kinase Inhibitor
CNS: Central Nervous System
Cys: Cysteine
DCC: Dicyclohexylcarbodiimide
DCM: Dichloromethane
DEA: Diethylamine
DEPT: Distortionless Enhancement by Polarisation Transfer
DIEA: N,N-Diisopropylethylamine
DMF: N,N-Dimethylformamide
DNA: Deoxyribonucleic acid
EB: Ethidium Bromide
ED-SCLC: Extensive Disease SCLC
EDT: Ethandithiol
EPR effect: Enhanced Permeability and Retention effect
ESIMS: Electrospray Ionisation Mass Spectrometry
ET-1: Endothelin
Fmoc: 9-Fluorenylmethyloxycarbonyl
GPCR: G-Protein Coupled Receptors
GRP: Gastrin Releasing Peptide
GRPR: Gastrin Releasing Peptide Receptor
HBTU: 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HF: Hydrogen Fluoride
i.p.: Intraperitoneal
i.v.: Intravenous
IGF-I: Insulin-like Growth Factor I
LD-SCLC: Limited Disease SCLC
Leu: Leucine
LPPS: Liquid Phase Peptide Synthesis
M: Metastasis
MALDIMS: Matrix Assisted Laser Desorption/Ionisation Mass Spectrometry
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>MBHA:</td>
<td>4-Methylbenzhydrylamine</td>
</tr>
<tr>
<td>MeCN:</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>MePhe:</td>
<td>N-Methyl Phenylalanine</td>
</tr>
<tr>
<td>Met:</td>
<td>Methionine</td>
</tr>
<tr>
<td>N:</td>
<td>Regional Lymph Node</td>
</tr>
<tr>
<td>NHS:</td>
<td>N-Hydroxysuccinimide</td>
</tr>
<tr>
<td>NMB:</td>
<td>Neromedin B</td>
</tr>
<tr>
<td>NMR:</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NSCLC:</td>
<td>Non-Small Cell Lung Cancer</td>
</tr>
<tr>
<td>NT:</td>
<td>Neurotensin</td>
</tr>
<tr>
<td>PC:</td>
<td>L-α-phosphatidylcholine</td>
</tr>
<tr>
<td>PDI:</td>
<td>Polydispersity Index</td>
</tr>
<tr>
<td>Phe:</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>PS:</td>
<td>Phosphatidyl serine</td>
</tr>
<tr>
<td>PyAOP:</td>
<td>(7-Azabenzotriazol-1-loxy)tripyrrolidinophosphonium hexafluorophosphate</td>
</tr>
<tr>
<td>Rb:</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>RP-HPLC:</td>
<td>Reversed Phase - High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>RT:</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>s.c.:</td>
<td>Subcutaneous</td>
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<tr>
<td>SCLC:</td>
<td>Small Cell Lung Cancer</td>
</tr>
<tr>
<td>SP:</td>
<td>Substance P</td>
</tr>
<tr>
<td>SPA:</td>
<td>Substance P antagonist A</td>
</tr>
<tr>
<td>SPD:</td>
<td>Substance P antagonist D</td>
</tr>
<tr>
<td>SPG:</td>
<td>Substance P antagonist G</td>
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<tr>
<td>SPPS:</td>
<td>Solid Phase Peptide Synthesis</td>
</tr>
<tr>
<td>T:</td>
<td>Tumour</td>
</tr>
<tr>
<td>t-BuOK:</td>
<td>Potassium tert-butoxide</td>
</tr>
<tr>
<td>TFA:</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>Tg:</td>
<td>Glass Transition Temperature</td>
</tr>
<tr>
<td>THF:</td>
<td>Tetrahydrofuran</td>
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<tr>
<td>TIS:</td>
<td>Triisopropylsilane</td>
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<tr>
<td>TLC:</td>
<td>Thin Layer Chromatography</td>
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<tr>
<td>tR:</td>
<td>Retention time</td>
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<td>Trp:</td>
<td>Tryptophan</td>
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List of Synthesised and Characterised Compounds

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<td>Boc-D-Trp(N-benzyl)-O-benzyl</td>
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<td>29</td>
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<td>DMePhe-DTrp(N-butyl)-Phe-DTrp-Leu-NH&lt;sub&gt;2&lt;/sub&gt;</td>
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**List of Synthesised and Characterised Compounds**

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<tr>
<td>Fmoc-D-Trp(N-tert-prenyl)-Leu-NH₂</td>
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List of Publications

The majority of the work presented in this thesis has been the subject of the following publications (Appendix II):

   (DOI: 10.1039/C7MD90009K)

   § *These authors contributed equally.*
   * Corresponding author.
   (DOI: 10.1039/C6MD00691D)

   * Corresponding author.
   (DOI: 10.1016/j.ejmech.2017.05.053)
Abstract

Small Cell Lung Cancer (SCLC) is an aggressive form of cancer accounting for 25% of lung cancer deaths worldwide. Treatment relies on combination chemotherapy (etoposide and cisplatin or carboplatin) with or without radiation therapy. However, disease relapse and resistance occurs quickly, prompting unmet need for alternative treatment options. One such option is the use of broad-spectrum antagonists, known as Substance P (SP) analogues. Historically, these analogues have not succeeded clinically due to low potency and bioavailability. In this project, novel SP analogues were developed to address these shortfalls. A chemical strategy was designed to synthesise novel short peptides including DMePhe-DTrp-Phe-DTrp-Leu-NH₂ (25) as the new lead. Fmoc and Boc D-Trp derivatives with indole nitrogen having substituents (methyl, ethyl, propyl, butyl, pentyl, propargyl, benzyl and tert-prenyl) were made and characterised by $^1$H and $^{13}$C NMR spectroscopy and mass spectrometry (MS). These building blocks were incorporated into the first series of peptides, substituting the D-Trp residue located near the C-terminal of 25, via solid and/or liquid phase procedures. Final products were purified by RP-HPLC to >90% purity and structures verified by MS and/or $^1$H NMR. Cell viability assays were conducted to evaluate cytotoxicity against two SCLC cell lines: H69 (chemo-naive) and DMS79 (from a patient after treatment). The IC$_{50}$ values for the D-Trp residue modified peptides were <5 µM. One of the earliest candidates to emerge from this work was DMePhe-DTrp-Phe-DTrp(N-tert-prenyl)-Leu-NH₂ (33). Subsequently, the most potent peptide was the one bearing D-Trp(N-butyl) (29) with IC$_{50}$ values of 1.0 µM (H69) and 1.4 µM (DMS79), compared to the lead 25 with IC$_{50}$ values of 30.7 µM (H69) and 23.0 µM (DMS79). A second series of peptides were produced to optimise 29 by incorporating a D-Trp(N-butyl) residue. The study focused on peptides by (a) modifying the N-terminal D-Trp residue, (b) modifying both D-Trp residues, (c) changing the C-terminal amide to free carboxylic acid, and (d) adding a charged amino acid (arginine) or removing a hydrophobic amino acid (leucine) to additionally aid in solubility. The most potent candidate was found to bear dual D-Trp(N-butyl) residues (35) with IC$_{50}$ value of 0.6 µM (H69) and 2.3 µM (DMS79). Peptides 29 and 35 were at least 26 times more potent than SP antagonist G (SPG, previously subjected to a Phase I clinical trial), as revealed by in vitro screening in this project. Both sequences induced apoptosis as evident from fluorescence staining. Flow cytometric analysis of 29 with the DMS79 cell line showed that the level of late apoptotic cells rose from 36% at 2 µM to 96% at 6 µM, compared to 25 that exhibited no effect. Efficacy of peptide 33 was separately evaluated in vivo using DMS79 xenografts. A low dose (1.5 mg/kg) was found to reduce tumour growth by ~30% (p < 0.05) at day 7, relative to the control group. Higher doses could not be used due to limited aqueous solubility. Furthermore, these peptides were shown to have improved stability. Exposed to neat mouse plasma for 48 hours, 29 and 35 remained intact by 68.5% and 81.0%, respectively, compared to 59.0% for 25 and 35.9% for 33. Complete metabolic stability of 29 and 35 was observed after 3 hours incubation in mouse S9 liver fraction. Aqueous solubility issues were overcome in feasibility studies incorporating 29 into liposomes for future in vivo efficacy testing. Finally, due to the high potency and stability of 29, a liposomal formulation of it may have a profound effect in in vivo efficacy studies against chemo-resistant SCLC.
Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.
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Special thanks go to my supervisor, Dr. Harmesh S. Aojula, without whom this work could not have been done. Thanks to his continuous academic guidance throughout the past three years. I also thank my co-supervisor, Dr. Sally Freeman, whom her knowledge and experience were invaluable. I learned a lot from both of them.

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I would like to thank Al-Zaytoonah University of Jordan for the PhD scholarship.

Finally, but not least, I would like to thank my father Haitham and my mother Yara for their continuous support and encouragements. Without them, I could not reach this level. I also thank my wonderful sister Anwaar for her moral support and loving feelings.
I dedicate this thesis to my wonderful family
Chapter 1

Introduction
1. Introduction
1.1 Lung Cancer
1.1.1 Aetiology
Lung cancer is the major cause of cancer related deaths in the UK (2014) accounting for 22%.\textsuperscript{1} There are several predisposing risk factors that lead to the cellular proliferation and development of lung cancer. Cigarette smoking is considered to have a strong association with lung cancer as most of lung cancer patients are smokers.\textsuperscript{2,3} In the UK, 83% of lung cancers are due to active smoking.\textsuperscript{1} The presence of tumour promoters and carcinogens in tobacco smoke are strongly linked to genetic mutations and abnormalities that lead to lung cancer.\textsuperscript{3-6} Smoking cessation, if maintained for more than 5 years, can decrease the risk of developing lung cancer but cannot reach the baseline level of non-smokers.\textsuperscript{3,4} There is also a link between passive smoking, in which non-smokers are exposed to tobacco smoke, with 15% of lung cancer cases reported in the UK.\textsuperscript{1} There is a linear correlation between smoke exposure and epithelial changes that with time leads to lung cancer.\textsuperscript{3}

There are also other risk factors contributing to the development of the disease. These include ionising radiation and miners/workers exposed to radioactive materials, asbestos, dusts containing cadmium, chromium, nickel, arsenic and uranium, vinyl chloride and mustard gas.\textsuperscript{3,7} Smoking along with these factors has a synergistic effect for developing lung cancer, especially with asbestos exposure.\textsuperscript{2,3}

Family history and specific genetic polymorphism, hereditary and mutations might increase the risk of developing lung cancer.\textsuperscript{3,8} For example, genetic polymorphism of the monooxygenase enzyme P-450 genes results in variable metabolising capacity of pro-carcinogens from tobacco, which in turn changes susceptibility for developing lung cancer.\textsuperscript{3} Mutations of proto-oncogenes, such as RAS, or mutations of tumour suppressor genes, such as TP53 are common in lung cancers.\textsuperscript{3,8} Overexpression of epidermal growth factor receptor (EGFR) and c-KIT receptor, amplifications of c-MET gene, and fusion of echinoderm microtubule-associated protein-like 4 (EML4) gene with anaplastic lymphoma kinase (ALK) gene to produce EML4-ALK protein
are also present in lung cancers. Other factors that increase the risk for lung cancer are patients with asthma and history of chronic obstructive diseases.

1.1.2 Clinical presentation and diagnosis

Patients who are suspected of having lung cancer show multiple signs and symptoms. These could be locally within the thorax, such as cough, dyspnoea, wheeze, hoarseness, chest, shoulder, or arm pain, tracheal obstruction, dysphagia, pleural effusion or superior vena cava obstruction. Other signs and symptoms occur due to metastasis, such as bone pain, liver dysfunction or neurological problems. Patients usually present with symptoms related to the region of metastasis rather than the lung tumour itself. Paraneoplastic syndromes, such as hypercalcemia, Cushing syndrome, syndrome of inappropriate secretion of antidiuretic hormone, anaemia, weight loss, nail clubbing, neuromuscular syndromes or hypercoagulable state, occur in 3-10% of lung cancer patients.

Their conditions are evaluated by visualising the tumour and by taking tissue samples for pathological assessment. Visualisation is mainly performed through chest radiographs that could show the tumour size, lymph node enlargement, any bone metastases within the chest area, pleural effusion and lobar collapse. Endobronchial ultrasound, CT and PET scans can also be utilised. The pathological assessment of the tumour can be identified by sputum cytology or mainly by performing tumour biopsy to identify histology and confirm malignancy. Biopsy is considered the optimal test for diagnosis and classification.

1.1.3 Morphological and histological classification

Lung cancer is broadly divided into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC).

NSCLC, which is diagnosed in 87% of lung cancers in the UK, is subdivided into three major types: squamous cell carcinoma, adenocarcinoma and large cell carcinoma. Squamous cell carcinoma, which represents less than 30% of lung
tumours, arises centrally (hilar) in major bronchi, strongly associated in patients with a long history of smoking and occurs more in men.\textsuperscript{2,4} Squamous cell carcinomas eventually spread to hilar nodes and outside the thorax and shows keratinisation.\textsuperscript{3} Adenocarcinoma, representing ~50\% of lung tumours, are mainly located in the periphery of the lung and considered the most common lung cancer in nonsmokers and women.\textsuperscript{3,4} Adenocarcinomas can occur in different histologic forms: 1) lepidic: mucinous that produce mucin, which is the main component of mucus, or non-mucinous cells that line the alveolar walls; 2) acinar (tumour arranged in acini and tubules - resembling glands); 3) papillary (papillae structures with fibrovascular core); 4) micropapillary (small papillae structures without fibrovascular core); and 5) solid types (nests or sheets of cells with mucin production).\textsuperscript{3,10} Large cell carcinomas are undifferentiated epithelial tumours and have large size located in the periphery of the lung.\textsuperscript{4}

SCLC, which is diagnosed in 12\% of lung cancers in the UK,\textsuperscript{1} is located centrally in the major bronchi and extends to lung parenchyma, hilar and mediastinal nodes.\textsuperscript{4} SCLC tumour cells are characterised to be small and fragile and have round-to-fusiform shape, scant cytoplasm, fine granular chromatin and absent or inconspicuous nucleoli.\textsuperscript{3,11}

1.1.4 Clinical classification

Lung tumours, including all its types, start with a local growth and then invades other tissues as metastasis occurs.\textsuperscript{2} The sites of metastases include regional lymph nodes, opposite lung, liver, bone, bone marrow, adrenal glands, kidneys, gastrointestinal tract and central nervous system (CNS).\textsuperscript{2,4} It is necessary to identify the clinical stage of a lung cancer patient to apply the optimum treatment regimen and predict the patient’s prognosis. The stages of lung cancer can be derived using tumour, node and metastasis (TNM) classification system adopted for NSCLC, which describes the size of tumour (T), regional lymph node involvement (N), and extent of metastasis (M) (summarised in Table 1).\textsuperscript{12} The same system is also used for SCLC classification.\textsuperscript{12}
Table 1. Tumour (T), regional lymph node (N) and metastasis (M) classification system. Table adapted from European Society for Medical Oncology guideline for SCLC diagnosis, treatment and follow-up. 

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<th>Description</th>
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<td>≤ 3 cm</td>
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<tr>
<td>T2:</td>
<td>&gt; 3 cm and &lt; 7 cm; main bronchus ≥ 2 cm from carina</td>
</tr>
<tr>
<td>T3:</td>
<td>&gt; 7 cm; chest wall, diaphragm, pericardium, mediastinal pleura, separate nodules in same lobe, total atelectasis (collapsed lung); main bronchus &lt; 2 cm from carina</td>
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<tr>
<td>T4:</td>
<td>Mediastinum, heart, great vessels, carina, trachea, esophagus, vertebra, separate nodules in different ipsilateral (one side of the lungs) lobes</td>
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<tr>
<td>N0:</td>
<td>No regional lymph node metastasis</td>
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<td>N1:</td>
<td>Ipsilateral peribronchial, ipsilateral hilar</td>
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<td>N2:</td>
<td>Ipsilateral mediastinal, subcarinal</td>
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<td>N3:</td>
<td>Supracalvicular, contralateral mediastinal or hilar</td>
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<td>No distant metastasis</td>
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<tr>
<td>M1:</td>
<td>Separate nodules in a contralateral lobe, pleural nodules, malignant pleural or pericardial effusions, distant metastasis</td>
</tr>
</tbody>
</table>

1.2 Small Cell Lung Cancer

SCLC accounts for approximately 25% of lung cancer deaths worldwide. It is more aggressive than NSCLC and considered a neuroendocrine tumour as it possesses several neuroendocrine markers. SCLC patients present with paraneoplastic syndromes more frequently than NSCLC patients.

1.2.1 Small Cell Lung Cancer clinical stages

SCLC patients are grouped into two clinical stages according to the TNM classification system: limited disease (LD) SCLC and extensive disease (ED) SCLC. LD-SCLC tumours are located within the hemithorax, the mediastinum and/or the supraclavicular lymph nodes, specifically T1-4/N0-3/M0 without distant metastasis, in which concurrent chemo-radiotherapy is used. ED-SCLC tumours
are presented with distant metastasis, T-Any/N-Any/M1,\textsuperscript{12} and treated with chemotherapy.\textsuperscript{13}

Median survival times vary among SCLC patients. From time of diagnosis, untreated SCLC patients have a survival time of 2-4 months,\textsuperscript{13} LD-SCLC patients treated with concurrent chemo-radiotherapy have a survival time of 14-20 months, and ED-SCLC patients treated with chemotherapy have a survival time of 9-11 months.\textsuperscript{4} The short survival times are attributed to the fact that although the disease is responsive to therapy, it usually recurs within 6 to 8 months with survival time becoming about 4 to 5 months.\textsuperscript{4}

\subsection*{1.2.2 Small Cell Lung Cancer cell lines}
Experimental \textit{in vitro} and \textit{in vivo} studies on SCLC are performed using various cell lines established from histologically identified SCLC patients who had been treated or untreated with chemotherapeutics or radiation therapy.\textsuperscript{16} These established cells retain very similar characteristics to the tumour from which they are derived.\textsuperscript{17}

SCLC and NSCLC cells can be clearly differentiated from one another in terms of growth and biochemical markers. In liquid cultures, most SCLC cells form suspension and grow as floating aggregates with tightly or loosely packed cells, whereas NSCLC cells are usually adherent cells.\textsuperscript{16, 17} SCLC overexpresses neuroendocrine biochemical markers that NSCLC rarely express. These markers include L-dopa decarboxylase (DCC), bombesin-like (BBN-like) peptides that include BBN and/or gastrin releasing peptide (GRP), neuron-specific enolase (NSE) and cytosolic isoform of brain-type creatine kinase (BB-CK).\textsuperscript{18-24} Hence, SCLC is termed as a neuroendocrine tumour.

L-dopa decarboxylase enzyme and neuron-specific enolase enzyme are both involved in glycolysis (metabolic pathway of glucose to release energy).\textsuperscript{17, 19} Both enzymes are normally found in amine precursor uptake and decarboxylase (APUD) series of neuroendocrine cells. APUD cells can form and store amines after the uptake and
subsequent decarboxylation of their precursor.\textsuperscript{25} Brain-type creatine kinase is a major enzyme that plays an important role in energy metabolism in non-muscle cells; it catalyses the reversible reaction of adenosine triphosphate (ATP) formation from the transfer of phosphoryl group from phosphorylcreatine (PCr) to adenosine diphosphate (ADP).\textsuperscript{24} Among others, bombesin and gastrin releasing peptide are neuropeptides that are present in CNS and in various tissues including endocrine tissues.

SCLC cell lines are further subdivided into two classes: classic and variant SCLC cell lines that correspond to classic and variant tumours. This is due to the heterogeneity of the expression of biomarkers within SCLC cell lines. Classic SCLC cell lines represent 70\% of SCLC cells with high expression of L-dopa decarboxylase, bombesin/gastrin releasing peptide, neuron-specific enolase and brain-type creatine kinase.\textsuperscript{17, 26} On the other hand, variant SCLC cell lines have high levels of neuron-specific enolase and brain-type creatine kinase but they lack L-dopa decarboxylase and/or bombesin/gastrin releasing peptide.\textsuperscript{17}

\textit{In vitro} and \textit{in vivo} variant SCLC cell lines grow more rapidly than classic cell lines.\textsuperscript{17} \textit{In vitro}, variant SCLC cell lines are more radio-resistant compared to classic cell lines.\textsuperscript{27, 28} Clinically, patients with variant SCLC tumours respond poorly to chemotherapy and have shorter survival time compared to patients with classic tumours.\textsuperscript{17, 28}

\textbf{1.2.3 Genetics in Small Cell Lung Cancer}

The development and progression of SCLC is attributed to several genetic abnormalities that lead to growth and cell cycle irregularities. The cell cycle and its regulatory proteins involved in SCLC mutations (described next) are presented in \textbf{Figure 1}. These abnormalities alter the functions performed by genes/proteins for regulating normal cell proliferation.
Figure 1. The cell cycle and the role of Rb, cyclin-CDKs complexes and CDKIs in its regulation at each phase. The figure shows the four phases of cell cycle (G1, S, G2 and M) and its two check points (G1-S and G2-M). The cyclin-CDKs complexes, which are inhibited by CDKIs, are involved in the regulation of the cell cycle at its check points and S phase. Mitogenic signals in tumours cause the phosphorylation of Rb (pRb) by the activated cyclin D-CDK4/6 complexes allowing cells to progress to S phase. Figure taken from Kumar et al. 29

Mutations of the tumour suppressor gene, TP53, is a major abnormality that occurs in >90% of SCLC. 3 The protein product of TP53, p53, is a transcription factor that regulates the transcription of several genes involved in different tumour suppressing cellular processes. 30 These processes include growth arrest at gap 1 (G1) presynthetic phase (for DNA repair), cellular senescence (permanent cell cycle arrest) and apoptosis induction. 30 The latter two would take place if DNA repair failed during G1 phase arrest. Cellular stresses, such as hypoxia and direct DNA damage are among several factors that cause p53 expression. 30 The G1 arrest is driven by the
transcription of cyclin dependent kinase inhibitor (CDKI) gene $CDKN1A$ to form $CDKN1A$ protein that inhibits cyclin–cyclin dependent kinase (CDK) complexes (described next in this section) (Figure 1). The cellular senescence also requires p53 and cyclin dependent kinase inhibitors expression. Apoptosis induction is mediated by p53 as it stimulates the transcription of pro-apoptotic gene ($BAX$) and $PUMA$ (regulates p53 function; described next in this section), to express BAX (pro-apoptotic protein) and PUMA proteins, respectively.

The transcription-independent induction of apoptosis caused by p53 is due to its interaction with B-cell lymphoma (BCL) family proteins (mitochondrial intrinsic pathway of apoptosis). The protein p53 releases the pro-apoptotic proteins (BAX and BAK) from their inhibitory complexes with anti-apoptotic proteins (BAX–BCL-XL and BAK–MCL-1). Another mechanism is the interaction of p53 with PUMA. In normal conditions, the amount of p53 is minimal in nucleus and cytosol and is kept inactive in the cytosol by being bound with BCL-XL. During cellular stresses, p53 levels increase and trigger PUMA expression, which will bind to BCL-XL and release p53 from the inhibitory complex (p53–BCL-XL). Then, p53 promotes the translocation of cytosolic BAX to mitochondria to initiate apoptosis.

Another major abnormality affecting tumour suppressor genes, which is present in ~90% of SCLC, is the mutation of the retinoblastoma ($RB$) gene. The protein product of $RB$, retinoblastoma (Rb), interacts with several proteins to regulate multiple cellular processes, such as the cell cycle, cell differentiation, DNA replication and repair, and apoptosis. Among the proteins that retinoblastoma interacts with, are those that belong to the E2F family. E2Fs are transcription factors which express several proteins that regulate the cell cycle at two check points: G1 phase to DNA synthesis/replication (S) phase and gap 2 (G2) pre-mitotic phase to mitotic (M) phase (Figure 1). E2Fs-mediated transcriptions express proteins called cyclins (cyclin E/D) that form complexes with cyclin dependent kinases (cyclin E–CDK2 and cyclin D–CDK4/6). These complexes activate cyclin dependent kinases to phosphorylate retinoblastoma allowing cells to progress from G1 phase to S phase.
Once cells reach S phase, they are committed to continue the cell cycle. To regulate the cell cycle, these complexes are inhibited by cyclin dependent kinase inhibitors. Among these are CDKN1A/1B/1C that inhibit all complexes while CDKN2A/2B/2C/2D selectively inhibit cyclin D–CDK4/6 complexes (Figure 1). During cellular stresses, the pathway would be as follows: inhibition of cyclin E–CDK2 complex by cyclin dependent kinase inhibitors (expressed due to p53 as described above), retinoblastoma becomes hypophosphorylated (active), and then active retinoblastoma prevents E2Fs mediated transcriptions resulting in cell cycle being in arrest for DNA repair. E2Fs mediated transcriptions are stopped by retinoblastoma forming complexes with E2Fs and/or recruiting chromatin remodelling proteins that make E2Fs-responsive genes insensitive to them. In tumours, the mitogenic signals would cause cyclin D expression which complexes with CDK-4/6 and hyperphosphorylate retinoblastoma (inactive form), thus inducing E2Fs mediated transcriptions followed by cell cycle progression. This role of retinoblastoma in cell cycle is presented in Figure 2.
Figure 2. The role of Rb in cell cycle at G1-S checkpoint. Figure representing the factors that hypophosphorylate and hyperphosphorylate Rb to regulate cell proliferation. Growth factors activate cyclin-CDKs complexes which phosphorylate Rb, causing the release of E2Fs to initiate their mediated transcriptions allowing cell cycle progression to S phase. Growth inhibitors stimulate CDKIs that inactivate cyclin-CDKs complexes, causing cell cycle to halt. Remodelling proteins (histone deacetylase and histone methyltransferase) cause E2Fs-responsive genes insensitive to them. Figure taken from Kumar et al.29

The other complexes present in cell cycle is cyclin A–CDK1/2, which are active in the S phase.29 The S phase genes transcriptional activation takes place when retinoblastoma is inactive and E2Fs are released. The complex that functions in another checkpoint (G2 to M) is cyclin B–CDK1.29 These are shown in Figure 1.

In cancers, mutations of one or more of the genes involved in retinoblastoma phosphorylation pathway are observed. Apart from RB gene, mutations of CDK4, cyclin D, and CDKN2A genes are present.29 Mutations of the tumour suppressor gene
CDKN2A occur in ~10% of SCLC.\(^3\) The protein product of CDKN2A, CDKN2A, is a cyclin dependent kinase inhibitor and functions as described above.

The mutations of the *Fragile Histidine Triad (FHIT)* gene, which is mainly due to chromosomal deletions on the short arm (p) of chromosome 3 where FHIT is present, are considered an early event among tumour suppressor genes abnormalities.\(^3,\)\(^9\) The protein product of FHIT, Fhit, is a hydrolase enzyme that cleaves diadenosine triphosphate (AP\(_3\)A) into ADP and adenosine monophosphate (AMP).\(^32\) Studies revealed that the bound form of Fhit with AP\(_3\)A, regardless of Fhit hydrolase activity, is considered an active tumour suppressor as it induces apoptosis.\(^32,\)\(^33\) This was elucidated using cells, with mutant Fhit that binds poorly to AP\(_3\)P, loosing its ability to induce apoptosis, in comparison to cells that maintained ability to induce apoptosis by having mutant Fhit that binds well to AP\(_3\)P with poor hydrolase activity.\(^33\) The 3p deletions are present in >90% of SCLC.\(^3\) FHIT deletions are present more in smokers than non-smokers and are even found at early stages in smokers without lung cancer.\(^3,\)\(^32\) This observation may show that FHIT is a target for tobacco carcinogens.\(^32\)

On the other hand, there are rare abnormalities of proto-oncogenes that develop in SCLC. The protein products of these genes normally promote cellular proliferation in a regulated manner. In tumours, these genes are described as oncogenes since they are a mutated or overexpressed form of proto-oncogenes.\(^29\) Among these oncogenes in SCLC are RAS and MYC.\(^3\)

RAS mutations are generally present in adenocarcinomas, but are rarely present in SCLC.\(^3\) There are three RAS types in humans: HRAS, KRAS2 and NRAS, with KRAS2 mutations being the common in lung cancers.\(^34\) The RAS products, RAS proteins, initiate cellular proliferation by stimulating downstream regulators via RAS-dependent kinase pathways.\(^29\) These regulators eventually initiate nuclear transcription of various genes. The active form of RAS is bound to guanosine triphosphate (GTP), while the inactive form is bound guanosine diphosphate (GDP).
Chapter 1

RAS proteins have intrinsic GTPase activity to hydrolyse GTP to GDP, and in doing so become inactive themselves. However, mutations that affect the GTP binding site (or the enzymatic region for GTP hydrolysis on RAS) cause the loss of the GTPase activity, keeping RAS in its activated form and resulting in continuous proliferation.

Amplifications of MYC family genes (CMYC, NMYC and LMYC) are among the mutations of oncogenes present in SCLC. The protein products of these genes act as transcription factors for several growth-promoting genes and as metabolism regulators. For example, MYC protein activates the transcriptions of CDKs and represses the transcription of CDKIs (functions described above). In cancer cells, upregulation of genes that promote aerobic glycolysis and increased utilization of glutamine are present and these are normally regulated by MYC. Hence, any MYC abnormality will promote cancer growth.

1.2.4 Factors attributed to Small Cell Lung Cancer resistance and relapse
Although SCLC is responsive to therapy initially, resistance occurs quickly and patients relapse. There are several predisposing factors and mechanisms that make SCLC resistant to current treatments.

The resistance to chemotherapy results from different factors. These include the genetic abnormalities, which dysregulate cell cycle, enhance proliferation, downregulate the pro-apoptotic proteins and others, and express several proteins. In addition, the presence of efflux P-glycoproteins and the amplifications of MYC genes are observed in relapsed patients. Other forms of chemotherapy resistance are the mutations or alteration of several metabolic enzymes.

Biopsies obtained from greater than 50% of newly diagnosed SCLC patients have tumours with hypoxic regions. Hypoxic cells are known to have increased resistance to chemotherapy and radiation therapy. Normally, the ionising radiations used in therapy produces free radicals that are stabilised or fixed permanently by
oxygen causing assured DNA damage.\textsuperscript{35} Resistance to radiation therapy in hypoxic cells arises as the free radicals produced by the ionising radiations are rapidly reduced preventing DNA damage.\textsuperscript{14} Hypoxic cells in tumours do not receive enough oxygen as they are far from the blood vasculature.\textsuperscript{14} Poor vasculature also hampers chemotherapeutics to reach those parts.\textsuperscript{14}

Resistance to treatment could also occur due to the presence of cancer stem cells (CSCs). CSCs are type of cell that have the capacity of self-renewal, proliferation, metastasis, tumour formation and resistance to chemotherapy and radiation therapy.\textsuperscript{36} In solid tumours, such as SCLC, they account for <1\% of total cells, in contrast to 27-100\% of total cells in highly tumourigenic cancers, such as haematopoietic and melanoma tumours.\textsuperscript{36} There are several glycoprotein cell markers that identify CSCs in lung cancers, such as CD56, CD90, CD44, CD105 and CD133.\textsuperscript{36} A study showed that SCLC cells that express CD133 are more resistant to etoposide chemotherapy than SCLC cells that do not express CD133.\textsuperscript{37} Furthermore, in a comparison between treated and untreated SCLC patients with conventional chemotherapy, tumour cells obtained after therapy expressed higher CD133 than those obtained before therapy.\textsuperscript{37}

1.2.5 Growth factors in Small Cell Lung Cancer

All normal cells require growth factors to stimulate their proliferation. Most of these growth factors are made in one cell type and act on a receptor in a neighbouring cell (paracrine action).\textsuperscript{29} On the other hand, several cancer cells, including SCLC cells, tend to overexpress several growth factors and their corresponding receptors, which together form autocrine growth loops (\textbf{Figure 3}).\textsuperscript{9, 29} Hence, enhancement of cell proliferation, mitogenesis and tumour development occurs. Additionally, several growth factors that bind to different receptors, stimulate intracellular signalling pathways, which act synergistically to promote cell growth and mitogenesis.\textsuperscript{38}
Autocrine growth loops and the mitogenic effect of growth factors were studied on Swiss 3T3 cells (Mouse Swiss Albino embryo fibroblast).\textsuperscript{39} These cells are a useful model to demonstrate the activity of growth factors, receptors and the signal transduction pathways responsible for initiation of cell growth. Their proliferation is halted when the nutrients become depleted in the cell culture medium. The cells would be either in G0 (cells that did not enter cell cycle) phase or G1 phase whereby their growth can be stimulated with the addition of fresh serum or growth factors. A number of the growth factors which acted as mitogens on Swiss 3T3 cells were also found to act as mitogens on SCLC cells.

Mitogenesis has been evaluated based on the ability of cells to stimulate DNA synthesis via $^3$H-thymidine incorporation assay.\textsuperscript{39, 40} Also, mitogenesis has been evaluated using cell proliferation assays (counting the number of living cells after being incubated with the growth factor) and/or measuring ions fluxes across the plasma membrane (effects observed from activating receptors).

Bombesin is a neuropeptide that was first isolated from frog.\textsuperscript{41} It has been used to study mitogeneis on Swiss 3T3 cells. Bombesin-like peptides have also been found in brain, gut and lung of mammals.\textsuperscript{42-45} Bombesin can stimulate the release of various peptide hormones, such as insulin, glucagon, gastrin, cholecystokinin (CCK),

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{schematic_representation}
\caption{A schematic representation for the autocrine growth loops present in SCLC. Growth factors being produced within the cell and bind on the receptor of the same cell.}
\end{figure}
prolactin and growth hormone. The importance of bombesin as a mitogen had risen since it induced pancreatic hyperplasia in rat. It was found that it acts on a specific receptor and release amylase when tested on pancreatic acinar cells from guinea pigs, rat and mouse.

Bombesin-like peptides were identified from porcine and are known as gastrin releasing peptide and neuromedin B (NMB), both of which are considered neuropeptides as they are present in the CNS and various tissues. They are the mammalian counterpart of bombesin. The amino acid sequences of the three neuropeptides have similarities in their C-terminal heptapeptide (Table 2). It was shown through DNA synthesis assays on Swiss 3T3 cells that the C-terminal heptapeptide of this family is responsible for the mitogenesis. Bombesin, gastrin releasing peptide, neuromedin B, gastrin releasing peptide-(14-27) and bombesin-(8-14) increased $^3$H-thymidine incorporation, in contrast to the N-terminal peptide sequence of gastrin releasing peptide [gastrin releasing peptide-(1-16)] (Table 2) that did not show any activity.

Table 2. Primary amino acid sequences of BBN, GRP, NMB, GRP-(1-16), GRP-(14-27) and BBN-(8-14). Similarities between sequences are in bold. pGlu: pyroglutamic acid; cyclic lactam of glutamic acid.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Primary Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBN</td>
<td>pGlu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$</td>
</tr>
<tr>
<td>GRP</td>
<td>Ala-Pro-Val-Ser-Val-Gly-Gly-Gly-Thr-Val-Leu-Ala-Lys-Met-Tyr-Pro-Arg-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$</td>
</tr>
<tr>
<td>NMB</td>
<td>$^{\text{Gly-Asn-Leu-Trp-Ala-Thr-Gly-His-Phe-Met-NH$_2$}}$</td>
</tr>
<tr>
<td>GRP-(1-16)</td>
<td>Ala-Pro-Val-Ser-Val-Gly-Gly-Gly-Thr-Val-Leu-Ala-Lys-Met-Tyr-Pro</td>
</tr>
<tr>
<td>GRP-(14-27)</td>
<td>Met-Tyr-Pro-Arg-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$</td>
</tr>
<tr>
<td>BBN-(8-14)</td>
<td>$^{\text{Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$}}$</td>
</tr>
</tbody>
</table>
The receptors of these neuropeptides belong to the G-protein coupled receptor (GPCR) family. On binding to G-protein coupled receptors, they initiate multiple signal transduction pathways to promote cellular proliferation. This eventually leads to multiple protein activation and expression to move cells from G0 or G1 to S phase for DNA synthesis. The important early events leading to mitogenesis following neuropeptides binding to their G-protein coupled receptors include: ion fluxes, protein kinase C (PKC) activation and induction of proto-oncogenes. These are summarised in Figure 4 and discussed next.
Figure 4. Early signal transduction pathways initiated by neuropeptides binding to GPCR which lead to cellular proliferation. PLC enzyme hydrolysies PIP2 into two second messengers: DAG and IP3, stimulating several signaling pathways for DNA synthesis.
The binding of the neuropeptide activates phospholipase C (PLC) enzyme to hydrolyse phosphatidyl inositol 4,5-bisphosphate (PIP$_2$) in the plasma membrane into two second messengers: inositol 1,4,5-triphosphate (IP$_3$) and 1,2-diacylglycerol (DAG). Inositol 1,4,5-triphosphate binds to ligand-gated Ca$^{2+}$ channel on the endoplasmic reticulum and releases the intracellular Ca$^{2+}$ stores into the cytosol. The increase in Ca$^{2+}$ cytosol concentration is one of the events involved in cells moving from G0 or G1 into S phase, as observed in SCLC cell lines when mitogens were tested. This is termed as Ca$^{2+}$ mobilisation; as Ca$^{2+}$ are mobilised from cellular internal stores into the cytosol. The other second messenger, 1,2-diacylglycerol, activates protein kinase C and protein kinase D, which phosphorylate other proteins in the cascade to drive cellular proliferation.

PKC phosphorylates myristoylated alanine-rich C kinase substrate (MARCKS/80k protein). The latter becomes downregulated as it becomes translocated to the cytosol. The protein 80k is a calmodulin and actin binding protein that is important in normal cellular development. It is highly expressed when cells exit the cell cycle and enter G0. Protein Kinase C is also involved in activating Protein Kinase D as well. Another role of protein kinase C is the activation of the amiloride-sensitive Na$^+$/H$^+$ antiport. The activation of this antiport increases the intracellular Na$^+$ levels in exchange for H$^+$ export. This in turn causes cytosolic alkalinisation. A secondary stimulation of Na$^+/K^+$ pump due to high Na$^+$ levels takes place to restore Na$^+$ electrochemical gradient by pumping K$^+$ outside the cell. The regulation of pH and K$^+$ to keep above critical threshold levels is important for stimulating cells from their G0 phase to next phases. Amplification of proto-oncogenes, such as $CMYC$ and $CFOS$ are also observed. Along with multiple signalling pathways stimulated, neuropeptides stimulate nuclear transcription, DNA synthesis and cell proliferation by binding to G-protein coupled receptors.

Bombesin is known to be expressed in SCLC. Gastrin releasing peptide and neuromedin B, along with their distinct receptors, are also present in SCLC cells. Acting as mitogens in SCLC, they stimulate Ca$^{2+}$ mobilisation and cell growth.
Gastrin releasing peptide and its receptor, gastrin releasing peptide receptor, seem to play a major role in SCLC development through an autocrine growth loop. Hence, gastrin releasing peptide receptor may be an important target for SCLC treatment. Gastrin releasing peptide is present in several normal tissues, such as in CNS, gastrointestinal tract, pancreas, thymus, prostate and pregnant uterus.\(^{20}\) Gastrin releasing peptide/neuromedin B are responsible for multiple roles in the body, such as gastric acid secretions, hormone release (gastrin, somatostatin and cholecystokinin), and smooth muscle contractions in stomach, small intestine and various other tissues.\(^{20}\) In addition, gastrin releasing peptide is detected in foetal and neonatal lungs, but is at a low level in normal adult lung.\(^{15,20}\) This implies its role in lung development at younger age.\(^{63}\) Hence, the gastrin releasing peptide elevation in plasma of patients with ED-SCLC could be involved in tumour growth enhancement.\(^{15,64}\) Studies showed that gastrin releasing peptide receptors become overexpressed in cell lines established after chemotherapy given to SCLC patients,\(^{37,56}\) but the increased expression of the receptor sensitises the cells further to Substance P (SP) analogues (discussed later) that inhibit SCLC cells growth.\(^{57,65}\)

There are also several other peptide growth factors shown to be mitogens of SCLC as elucidated from Ca\(^{2+}\) mobilisation and cell growth assays. Their mitogenic effect is also due to the co-expression of the peptides and their distinct receptors, which form autocrine growth loops. These include: (neuropeptide/receptor) bradykinin (BK)/BK-B2, arginine vasopressin (AVP)/V1a, cholecystokinin /CCK-A and CCK-B and neurotensin (NT)/NTR.\(^{58,66-68}\) Other peptides that may act as growth factors in SCLC include galanin, transferrin, vasoactive intestinal peptide and opioid peptides.\(^{15,69}\) Apart from neuropeptides, autocrine growth loops in SCLC could also occur as a result of the co-expression of peptide hormones and their receptors, such as gastrin/CCK-B, endothelin (ET-1)/ETAR and ETBR, insulin-like growth factor I (IGF-I)/IGF-I receptor and stem cell factor/c-KIT.\(^{15,58,70-72}\) It is worth mentioning that the existence of autocrine growth loops is characteristic of SCLC rather than NSCLC.\(^{58}\)
1.3 Current management of Small Cell Lung Cancer

Each clinical stage of SCLC has its own treatment regimen, which depends on the tumour stage classified from the TNM system. These treatment regimens include surgery, intravenous (i.v.) or oral chemotherapy, and thoracic radiation therapy.

1.3.1 Limited Disease - Small Cell Lung Cancer

LD-SCLC patients (T1-4/N0-3/M0) receive concurrent thoracic radiotherapy and i.v. combination chemotherapy as first-line treatment. The combination chemotherapy consists of etoposide with platinum analogue, preferably cisplatin. Carboplatin may cause increased thrombocytopenia, but can be substituted for cisplatin to decrease nephrotoxicity, neurotoxicity, or gastrointestinal toxicity. A prophylactic cranial irradiation (PCI) is given after a successful first-line treatment. The reason for PCI is to decrease the risk of brain metastasis and improve survival.

However, LD-SCLC patients that are diagnosed with T1,2/N0,1/M0 can undergo a surgical resection, if there is no perioperative complications, followed by adjuvant combined chemotherapy.

1.3.2 Extensive Disease - Small Cell Lung Cancer

The first-line treatment of ED-SCLC patients (T-Any/N-Any/M1) is i.v. combination chemotherapy consisting of etoposide and cisplatin. However, a meta-analysis of six trials involving 1476 untreated Asian and white patients with ED-SCLC showed better response rates and overall survival using irinotecan and cisplatin rather than etoposide and cisplatin regimen. Moreover, Asian clinical trials involving irinotecan and cisplatin resulted in lower rates of toxic effects and death in comparison to European or US trials involving the same regimen. Hence, Asian patients with ED-SCLC receive the irinotecan and cisplatin regimen. PCI is given after a successful first-line treatment.

ED-SCLC patients, who are young or with localized disease, are recommended to take etoposide with cisplatin. In cases where etoposide is contraindicated, the
following regimens could be an alternative: irinotecan with cisplatin, gemcitabine with carboplatin or topotecan with cisplatin.\textsuperscript{12}

\subsection*{1.3.3 Relapsed Small Cell Lung Cancer}
Due to the aggressive nature of SCLC, first-line treatment eventually fails and disease relapses. If the disease relapses within 3 months after ending the first-line treatment regimen or patient is not responding during the chemotherapy, the patient is classified to have resistant or refractory SCLC, respectively, and the available options would be either supportive care or clinical trials enrollment.\textsuperscript{4, 9, 12} Second-line chemotherapy treatment is considered for patients with disease relapse occurring after 3 months of ending first-line treatment.\textsuperscript{4} Those patients are classified to have sensitive SCLC.\textsuperscript{9, 78} Topotecan (i.v. or oral) is administered as the second-line treatment. Patients with sensitive SCLC may benefit from re-prescribing first-line treatment regimen for them as well.\textsuperscript{9, 12} However, the benefit of second-line treatment is short-lived and survival time is only few months longer.\textsuperscript{9}

\subsection*{1.3.4 Treatments toxicity}
As any other medication regimen, chemotherapy and radiotherapy have side effects. Chemotherapy could result in haematological toxicity or myelosuppression (anaemia, neutropenia and thrombocytopenia), nephrotoxicity, neurotoxicity, gastrointestinal toxicity (nausea, vomiting and diarrhoea), hypotension and alopecia (hair loss).\textsuperscript{9, 12, 79} Radiotherapy can damage normal tissues and could result in patients getting acute oesophagitis and neurotoxicity.\textsuperscript{9, 80} Cranial irradiation could cause fatigue, hair loss and neurocognitive side-effects.\textsuperscript{12}

\subsection*{1.4 Investigating different therapeutic approaches for Small Cell Lung Cancer treatment}
Radiation therapy causes single and double strand DNA breaks, in which the latter is considered the main cause of cell death.\textsuperscript{35} DNA damage will affect several cellular functions, such as DNA repair, cell cycle, gene expressions and apoptosis, which will collectively lead to cell death.\textsuperscript{35} Radiation causes DNA damage in two ways, either
directly by acting on DNA itself or indirectly via the formation of free radicals due to the ionisation of water components of the cell.  

Etoposide, irinotecan, topotecan, platinum analogues (cisplatin and carboplatin) and gemcitabine cause their anti-tumour effect through several mechanisms of action. Etoposide inhibits topoisomerase II enzyme; the enzyme which cuts both DNA strands to enable DNA replication or transcription. Topoisomerase I enzyme, which cuts a single DNA strand, is inhibited by irinotecan and topotecan. The platinum analogues (cisplatin or carboplatin) form intra-strand and inter-strand DNA cross-links and bind to nuclear and cytoplasmic proteins. Gemcitabine inhibits several enzymes required for DNA synthesis and repair.

The previous therapies are considered as non-targeted therapy for SCLC. For example, the aforementioned chemotherapeutics can be used in several other cancers, such as NSCLC, colorectal cancer, ovarian cancer, breast cancer, bladder cancer, gastroesophageal cancer, pancreatic cancer. Radiation therapy can be used to treat lymphomas and cancers of head, neck, prostate, breast and bladder.

1.4.1 Targeted therapies for the potential treatment of Small Cell Lung Cancer

Although SCLC patients respond well to the current chemotherapy and radiation therapy, relapse and resistance to therapy develop shortly. This explains the low survival time of SCLC patients. The toxicity of chemotherapeutics and radiation therapy and the development of resistance have led to investigate different options for the potential treatment of SCLC.

Studies have focused at the cellular and molecular level, of the pathogenesis of SCLC, to develop novel therapeutic agents. These therapies include (among others): targeting proliferative pathways, targeting apoptotic pathways, anti-angiogenic strategies and immunotherapy. There are agents that had undergone preclinical trials. Many have failed clinical trials, some showed a little improvement and others are still being tested or investigated.
The development of compounds that inhibit several proliferative signaling pathways in SCLC are being investigated and tested. These compounds range from selective compounds that inhibit certain receptors and pathways to compounds that target multiple receptors, such as Substance P analogues (discussed in detail in the next section).

Targeting receptor tyrosine kinases, which includes c-Kit, c-Met, epidermal growth factor receptor, insulin-like growth factor-1 receptor, fibroblast growth factor receptors and vascular endothelial growth factor receptors have been studied. As insulin-like growth factor-1 acts as an autocrine growth factor in SCLC and its receptor is over expressed in SCLC, an antibody called cixutumumab targeting the receptor is currently in Phase II trial combined with etopside and cisplatin.

In SCLC, simvastatin was active in vitro and in vivo, and showed to reduce activation of signaling pathways activated by Ras proteins. Although a Phase II trial including a combination therapy of simvastatin with irinotecan and cisplatin failed to improve survival of ED-SCLC patients, there are still ongoing Phase III trial using pravastatin with etoposide and cisplatin.

The intracellular signaling pathway, PI3K/Akt/mTOR, is usually deregulated and activated in several cancers including SCLC. There are several inhibitory agents are currently being investigated in Phase I trials, such as temsirolimus, and others being terminated, such as everolimus.

Hedgehog pathway is another proliferative pathway that is reported to cross-talk with the pathway activated by bombesin in SCLC. There are several hedgehog pathway inhibitors, such as GDC-0449, are being investigated in Phase I and II trials.

Poor survival in SCLC patients was found to be correlated with the expression of vascular endothelial growth factor, which is an important angiogenic factor. Bevacizumab, a monoclonal antibody targeting vascular endothelial growth factor-A,
was suspended after several clinical trials. In Phase II trials, bevacizumab improved the overall survival with the addition of irinotecan and cisplatin, but no similar improvement was shown with the addition of cisplatin or carboplatin plus etoposide. Sunitinib, a broad spectrum tyrosine kinase inhibitor (vascular endothelial growth factor receptor is a tyrosine kinase receptor), is currently undergoing Phase I and II trials.

There are several inhibitors targeting the anti-apoptotic proteins (Bcl-2 proteins) that have been investigated in Phase I and II trials, but there are no current clinical trials being investigated.

Ipilimumab, a monoclonal antibody directed against cytotoxic T lymphocyte-associated protein 4, had been evaluated in Phase II trial in ED-SCLC patients and showed efficacy when combined with other chemotherapy. There are still ongoing Phase II and III trials using ipilimumab for SCLC treatment.

Targeting the enzyme Poly-(ADP-ribose) polymerase (PARP1), which is a DNA repair protein, showed efficacy in pre-clinical SCLC models. Phase I and II trials using PARP inhibitors, such as veliparib and alisertib, are currently being initiated for the treatment of SCLC.

Other agents that are currently being investigated in clinical trials for the treatment of SCLC include histone deacetylase (enzyme involved in chromatin modification) inhibitors and heat shock proteins inhibitors.

1.4.2 Role of Substance P analogues in Small Cell Lung Cancer

1.4.2.1 Substance P

The undecapeptide Substance P [Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂] was first isolated from alcoholic extracts of different tissues from horses. It possesses a pharmacological action as a vasodilator and decreases blood pressure on an atropinized rabbit.
Substance P is part of the tachykinin family found in mammals and in humans. It is a neurotransmitter that is found in the central and peripheral nervous systems.\textsuperscript{103,104} It acts through tachykinin (neurokinin NK) receptors (NK\textsubscript{1}, NK\textsubscript{2} and NK\textsubscript{3}) and mainly on NK\textsubscript{1} receptor.\textsuperscript{104} The binding of Substance P to its receptor initiates several signal transduction pathways. These pathways include the stimulation of adenylate cyclase enzyme to produce cAMP, stimulation of phospholipase C for phosphatidyl inositol turnover and stimulation of phospholipase A2 for arachidonic acid mobilisation.\textsuperscript{105}

When released from efferent neurons, Substance P causes different actions on several tissues, such as blood vessels, respiratory tract, gastrointestinal tract and genitourinary tract. These actions include: vasodilation, plasma extravasation, bronchoconstriction (activation of NK\textsubscript{1} receptors in humans), broncodilation (activation of NK\textsubscript{1} receptors in guinea-pig) and gland secretions in respiratory tract, regulation of fluid secretions in the gut, and smooth muscle contraction in the gut and genitourinary tract that increases motility.\textsuperscript{104}

Substance P and its receptor NK\textsubscript{1} are also present in several tumour cells, including lung tumours (SCLC and NSCLC), and in intra- and peri-tumoural blood vessels.\textsuperscript{105} The overexpression of NK\textsubscript{1} receptors is a characteristic marker in malignant tissue compared to benign tissue.\textsuperscript{106-108} The presence of Substance P and its interaction with the overexpressed NK\textsubscript{1} receptors in tumours is involved in variety of functions. In tumour cells, Substance P induces cell proliferation and apoptosis inhibition by activating different enzymes.\textsuperscript{108-112} Substance P also causes the migration of tumour cells through changing their cellular shape by membrane blebbing and expression of matrix metalloproteinases.\textsuperscript{113,114} Blebbing and metalloproteinases, which degrade extracellular matrix proteins, are responsible for metastasis and cancer progression. Substance P also induces angiogenesis as it interacts with NK\textsubscript{1} receptors expressed on intra- and peri-tumoural blood vessels.\textsuperscript{115}
1.4.2.2 The approach leading to the synthesis of Substance P analogues for Small Cell Lung Cancer treatment

As described earlier, there are several neuropeptides that act as growth factors in SCLC and work on different receptors. This led to the discovery of synthesis of broad-spectrum neuropeptide antagonists, known as Substance P analogues, which act on several cell-surface receptors. They are shown to be more potent than receptor-specific therapies for inhibiting the growth of SCLC. 

Although the name might suggest that these analogues act directly on Substance P receptors; in fact, they act on several receptors. As a pharmacological tool, the synthesis of these peptides was initially based on neurokinins (Substance P, neurokinin A and neurokinin B) to inhibit their actions. The modifications applied to the Substance P sequence were changing the isomer of specific amino acids, using different amino acids and/or shortening the peptide sequence.

Substance P antagonist A (SPA) [DArg-DPro-Lys-Pro-Gln-Gln-DTrp-Phe-DTrp-Leu-Leu-NH₂] was amongst the first Substance P analogues to be investigated for its anti-tumour activity. It was first synthesised and studied on isolated guinea-pig ileum and rat urinary bladder. Substance P antagonist A inhibited the Substance P-induced contractions of the isolated tissues and blocked Substance P receptors competitively, hence, it is called Substance P antagonist. Other experiments using Substance P antagonist A also revealed its ability to inhibit the bronchoconstriction and vascular permeability of trachea and oesophagus in guinea pig caused by Substance P. Substance P antagonist A also inhibited the contractile effect induced by SP on isolated guinea-pig taenia coli. In pancreatic acinar cells from guinea pig, Substance P antagonist A was shown to be a competitive Substance P and bombesin receptors antagonist and inhibited bombesin-stimulated amylase release.

On Swiss 3T3 cells, a model used to study mitogenesis of bombesin, bombesin-like peptides and other growth factors, Substance P antagonist A inhibited gastrin
releasing peptide and arginine vasopressin binding and mitogenesis induced by bombesin, gastrin releasing peptide and arginine vasopressin.\textsuperscript{50, 120, 121}

Substance P had no mitogenic effect on Swiss 3T3 cells and did not prevent the inhibitory action of Substance P antagonist A on bombesin-induced mitogenesis.\textsuperscript{120} In pancreatic acinar cells, Substance P antagonist A had 2500-fold lower affinity towards Substance P receptor than Substance P and of much higher affinity towards bombesin receptor than Substance P.\textsuperscript{119} Moreover, Substance P did not inhibit gastrin releasing peptide binding and did not stimulate DNA synthesis on Swiss 3T3 cells.\textsuperscript{39, 50} This might suggest that Substance P antagonist A acts, with high affinity, through bombesin or bombesin-like peptides receptors and not only through Substance P receptors. In addition, Substance P antagonist A acts on several receptors rather than a single one; Substance P antagonist A inhibited the mitogenesis induced by gastrin releasing peptide and arginine vasopressin, both of which have been shown to act on different receptors as concluded from binding assays using labelled and unlabelled gastrin releasing peptide and arginine vasopressin.\textsuperscript{121} In that binding assay, labelled gastrin releasing peptide binding was inhibited by unlabelled gastrin releasing peptide but not with unlabelled arginine vasopressin, and labelled arginine vasopressin binding was inhibited by unlabelled arginine vasopressin but not with unlabelled gastrin releasing peptide. Hence, Substance P antagonist A is considered a broad-spectrum neuropeptide antagonist.

Mitogenesis on Swiss 3T3 cells in the presence of Substance P antagonist A was also evaluated by monitoring the signal transduction pathways initiated by bombesin, gastrin releasing peptide and arginine vasopressin binding to their receptors. Substance P antagonist A inhibited a protein (80k) phosphorylation that normally occurs with bombesin or gastrin releasing peptide activating protein kinase C.\textsuperscript{122} Substance P antagonist A also inhibited intracellular pH increase and intracellular Ca\textsuperscript{2+} mobilisation that normally occurs with bombesin and arginine vasopressin.\textsuperscript{121, 123}
1.4.2.3 Anti-tumour activity of Substance P analogues in Small Cell Lung Cancer

Taking into consideration the: a) inhibitory effect of Substance P antagonist A on Swiss 3T3 mitogenesis caused by bombesin, gastrin releasing peptide and arginine vasopressin, b) the presence of bombesin and bombesin -like peptides in SCLC,\textsuperscript{21-23} and their mitogenic effect on SCLC cell lines,\textsuperscript{59, 60} further Substance P analogues were developed and their potential use for the treatment of SCLC was investigated. Table 3 shows the primary amino acid sequences of the Substance P analogues which have been previously synthesised and shown to have promising anti-tumour activity against SCLC, as discussed below.

<table>
<thead>
<tr>
<th>Table 3. Primary amino acid sequences of SP and SP analogues. Modifications applied to the SP sequence are in bold. MePhe: methyl phenylalanine; MPA: 2-amino-4-methylpentane; Leuψ(CH\textsubscript{2}NH)Leu: two leucine residues attached with reduced amide bond; pHOPA: para-hydroxy-phenyl-acetyl.</th>
</tr>
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<tbody>
<tr>
<td>SP: Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH\textsubscript{2}</td>
</tr>
<tr>
<td>SPA: DArg-DPro-Lys-Pro-Gln-Gln-DTrp-Phe-DTrp-Leu-Leu-NH\textsubscript{2}</td>
</tr>
<tr>
<td>SPD: DArg-Pro-Lys-Pro-DPhe-Gln-DTrp-Phe-DTrp-Leu-Leu-NH\textsubscript{2}</td>
</tr>
<tr>
<td>SPG: Arg-DTrp-MePhe-DTrp-Leu-Met-NH\textsubscript{2}</td>
</tr>
<tr>
<td>NY3238: pHOPA-DTrp-Phe-DTrp-Leu-Leu-NH\textsubscript{2}</td>
</tr>
<tr>
<td>NY3460: DMePhe-DTrp-Phe-DTrp-Leuψ(CH\textsubscript{2}NH)Leu-NH\textsubscript{2}</td>
</tr>
<tr>
<td>NY3521: DMePhe-DTrp-Phe-DTrp-Leu-MPA</td>
</tr>
<tr>
<td>[D-Arg\textsuperscript{1},D-Trp\textsuperscript{5,7,9},Leu\textsuperscript{11}]SP:</td>
</tr>
<tr>
<td>DArg-Pro-Lys-Pro-DTrp-Gln-DTrp-Phe-DTrp-Leu-Leu-NH\textsubscript{2}</td>
</tr>
<tr>
<td>[D-Arg\textsuperscript{1},D-Trp\textsuperscript{5,7,9},D-Leu\textsuperscript{10},Leu\textsuperscript{11}]SP-OH:</td>
</tr>
<tr>
<td>DArg-Pro-Lys-Pro-DTrp-Gln-DTrp-Phe-DTrp-DLeu-Leu-OH</td>
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In a cell proliferation assay, Substance P (100 µM) showed no enhancement or inhibitory effect on H69 and H345 cell lines after 8 and 11 days, respectively.\textsuperscript{124} This is similar to the previous\textsuperscript{120} results that showed it has no mitogenic effect on Swiss 3T3 cells. However, in two separate MTS cell viability assays, Substance P induced
the proliferation of H69 cells after 24 hours with maximum level (14% and 37%; compared to controls) reached by as little as 10 nM.\textsuperscript{125,126} This might be in line with the expected role of Substance P in tumour development, as described earlier, but rather at very low concentrations.

On H69 cell line, Substance P antagonist A and Substance P antagonist D caused a concentration dependent growth inhibition through cell proliferation assay. The half-maximal growth inhibition after 12 days was seen at 24 µM for Substance P antagonist D and at 82 µM for Substance P antagonist A.\textsuperscript{127} On various SCLC cell lines, half-maximal inhibition of DNA synthesis was achieved by 20 to 30 µM Substance P antagonist D after 24 hours.\textsuperscript{128}

On H69 cell line, Substance P antagonist D was shown to exert its anti-proliferative effect, in part, through blocking NK\textsubscript{1} receptors.\textsuperscript{126} This was clear as nanomolar concentration of Substance P (10 nM), which acts selectively on NK\textsubscript{1} receptors, competitively inhibited Substance P antagonist D cytotoxicity. This was elucidated as lower cellular proliferation was observed using Substance P antagonist D without Substance P. On the same cell line, Substance P antagonist D had IC\textsubscript{50} value of 51.05 µM found through MTS cell viability assay after 24 hours treatment.\textsuperscript{126} In addition, the cytotoxic effect of Substance P antagonist D was also observed by its ability to induce apoptosis.\textsuperscript{126,129}

Substance P antagonist D was tested \textit{in vivo} using HC12 and ICR-SC112 tumour-bearing mice.\textsuperscript{130} Intraperitoneal (i.p.) administration of Substance P antagonist D was not effective in comparison to the continuous subcutaneous (s.c.) peritumoural administration over a period of 14 days with a dose of 2.1 µg/day. A significant tumour volume reduction was achieved after 14 days compared to control animals with % inhibition reaching 75 ± 12% for HC12 xenografts and 61 ± 4% for ICR-SC112 xenografts. Monitoring HC12 and ICR-SC112 xenografts for further 14 and 21 days, respectively, growth rate was found similar to that of the controls (no
further growth inhibition), but reduced tumour volume was still observed (HC12: 67 ± 12%; ICR-SC112: 53 ± 6%) without rebound growth.

Following s.c. injection of 100 µg/g/day of Substance P antagonist D for 7 days on WX322 xenografts-bearing mice, a significant growth inhibition was observed when compared to controls at day 7. The same dose at the time of tumour implantation also caused significant growth inhibition.

On H69 and H345 cell lines, Substance P antagonist D and Substance P antagonist G, tested at 100 µM in a proliferation assay, were found to be the most potent peptides (among others) to inhibit the growth of cells after 8 (H69) and 11 (H345) days. Substance P antagonist D and Substance P antagonist G at 10 µM were also able to inhibit Ca²⁺ mobilisation induced by bradykinin, arginine vasopressin, gastrin releasing peptide, neurotensin, galanin or cholecystokinin in H69, H345 and H510A cell lines.

In a different study, Substance P antagonist G had IC₅₀ values of 24.5 ± 1.5 µM and 38.5 ± 1.5 µM on H69 and H510 cell lines, respectively, found through proliferation assays after 9 days. The study also showed that the cytotoxicity of Substance P antagonist G is driven by apoptosis induction. Other MTT cell viability assays were conducted on SCLC cell lines. They showed that Substance P antagonist G had IC₅₀ values of 129 ± 7.42 µM and 39.3 ± 19.5 µM on H69 and H82 cell lines, respectively, after 48 hours treatment.

Substance P antagonist G was tested in vivo on H69 xenograft-bearing mice. A daily peritumoural s.c. injection of 0.9 mg/day for 7 days, significantly inhibited tumour growth in comparison to controls. A significant inhibitory effect was maintained for at least 7 days after Substance P antagonist G administration stopped.

Substance P antagonist G was also evaluated in vivo in two different xenografts models with s.c. and i.p. injections. A peritumoural s.c. injection of 45 µg/g/day for 7
days on WX322 and H69 xenografts-bearing mice resulted in significant growth inhibition and significant tumour volume reduction which was maintained after stopping treatment for at least 28 and 7 days, respectively. The same dose was administered i.p. and resulted in a smaller inhibitory effect on both xenografts, but significant inhibitory effect was observed at day 7 on WX322 xenografts.

Compared to the undecapeptides (Substance P antagonist A and Substance P antagonist D), the shorter hexapeptide Substance P antagonist G was able to show in vitro and in vivo efficacy on SCLC. Further shorter Substance P analogues were synthesised and investigated for their effect on SCLC. These include the hexapeptide NY3460 and the pentapeptide NY3521. NY3460 and NY3521 were developed from NY3238; a known Substance P antagonist tested on guinea-pig ileum.

In a cell proliferation assay, NY3238, NY3460 and NY3521 inhibited H69 cell growth for a period of 12 days using 50 µM of each peptide. NY3460 also caused a decrease in cell number compared to the other two peptides. Studying their anti-mitogenic effect on H69 cell line, ³H-thymidine incorporation assay was conducted. The half-maximal inhibition of DNA synthesis was achieved with ~3 µM of NY3460 and NY3521, while it was ~15 µM for NY3238 after 3 days of peptides treatment.

MTT Cell viability assays conducted on several SCLC cell lines, showed that NY3460 had IC₅₀ values in the range of 2.8 to 3.7 µM after 5 days of treatment and induced apoptosis.

NY3460 was injected peritumourally (s.c.) at a dose of 45 µg into H69 xenografts-bearing mice. The dose was given on days at which tumour volume was measured (9 measurements over ~20 days) and showed significant growth inhibition when compared to controls.

[D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]Substance P is another undecapeptide Substance P analogue that was synthesised among many peptides and showed highest cytotoxicity against SCLC. The new peptides were based on Substance P antagonist D and Substance
P antagonist G sequences. When the activity of the peptides were compared to Substance P antagonist D and Substance P antagonist G in a cell proliferation assay for 12 days at 25 µM, Substance P antagonist G, Substance P antagonist D and [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]Substance P resulted in 30%, 61% and 92% growth inhibition, respectively. As [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]Substance P was the most potent peptide, it was selected for further experiments. For a period of at least 15 days in a proliferation assay using 20 µM of [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]Substance P, the growth of H69 and H510 cells was greatly reduced (> 80%) when compared to the controls. Similar results were obtained using various [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]Substance P concentrations in a cell proliferation assays for 12-14 days. The ability of the new analogue to inhibit mitogenesis in terms of intracellular Ca²⁺ mobilisation was also evaluated on H510 cells.

In H69 xenograft-bearing mice, a peritumoural s.c. injection of 35 µg/g/day for 7 days of [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]Substance P resulted in significant growth inhibition when compared to controls. The growth inhibition lasted for 23 days from injection, with 4 of 8 animals showing a disappearance of the tumours. The i.p. administration of the analogue for 7 days using a dose of 35 µg/g twice daily, on H69 xenograft-bearing mice, resulted in lower inhibition than s.c. administration, but nevertheless was still significant.

[D-Arg¹,D-Trp⁵,⁷,⁹,D-Leu¹⁰,Leu¹¹]Substance P-OH is the most recent Substance P analogue to be synthesised and investigated for SCLC treatment in vitro and in vivo. The differences between this and its predecessor, [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]Substance P, is that it has a free carboxylic acid in its C-terminal instead of an amide and a D-Leu instead of Leu at position 10. On H69 and H345 cell lines, MTT cell viability assays showed that [D-Arg¹,D-Trp⁵,⁷,⁹,D-Leu¹⁰,Leu¹¹]Substance P-OH has IC₅₀ values of 4.96 µM and 3.06 µM, respectively, after 3 days treatment. On the same cell lines, Substance P antagonist G has IC₅₀ values of 15.53 µM (H69) and 11.87 µM (H345). On H345 xenograft-bearing mice, a total of 5 peritumoural s.c. injections (25 µg/g each) showed a significant
growth inhibition for the duration of the study (33 days) compared to controls, with 3 of 6 mice showing a complete response.\textsuperscript{37}

1.4.2.4 Stability of Substance P analogues

\emph{In vitro} and/or \emph{in vivo} metabolic stability studies were conducted on SP and some of the previously mentioned Substance P analogues (Substance P antagonist G, Substance P antagonist D and [D-Arg\textsuperscript{1},D-Trp\textsuperscript{5,7,9},D-Leu\textsuperscript{10},Leu\textsuperscript{11}]Substance P-OH).

In a radioimmunoassay, the analysis of plasma obtained from dogs injected with Substance P showed a rapid degradation of Substance P with half-life ($t_{1/2}$) of 1.8-2.1 minutes.\textsuperscript{138} The study also suggested that the degradation of Substance P was mainly on its C-terminal.\textsuperscript{138}

The stability of Substance P antagonist G was studied using samples analysed from plasma, S9 liver preparation, and homogenates of liver and H69 xenograft.\textsuperscript{37, 139, 140} Substance P antagonist G had three major metabolites being identified: de-amidated- Substance P antagonist G (terminal amide was changed to carboxylic acid), oxidised-de-amidated- Substance P antagonist G (Met was oxidised) and Substance P antagonist G -minus-Methionine (Met-NH\textsubscript{2} was removed). Substance P antagonist G had a $t_{1/2}$ of 28.9 minutes in plasma and a $t_{1/2}$ of 9.32 minutes in S9 liver preparation.\textsuperscript{37, 140}

Substance P antagonist D stability was studied \emph{in vitro} using liver homogenate and was compared to Substance P antagonist G.\textsuperscript{141} The metabolism for Substance P antagonist D resulted in 2 major metabolites: de-amidated- Substance P antagonist D (terminal amide changed to carboxylic acid) and Substance P antagonist D -minus-Leucine (Leu-NH\textsubscript{2} was removed). A 50 nmol of Substance P antagonist D incubated for 1 hour showed that ~62% of it remained intact. However, the same experiment was conducted on Substance P antagonist G showing that the amount remaining intact was ~42%.
As in Substance P, the C-terminal of Substance P antagonist G and Substance P antagonist D is the site prone to degradation rather than the N-terminal. It was also shown that the Substance P antagonist G metabolites were less potent than Substance P antagonist G by at least 2 times in terms of inhibiting arginine vasopressin-stimulated growth in Swiss 3T3 cells (derived from ³H-thymidine incorporation assay).¹³⁹ Likewise, Substance P antagonist D metabolites did not maintain Substance P antagonist D antagonist effects of inhibiting bombesin, arginine vasopressin or bradykinin stimulated growth evident from similar assay on Swiss 3T3 cells.¹⁴¹

[D-Arg¹,D-Trp⁵,⁷,⁹,D-Leu¹⁰,Leu¹¹]Substance P-OH was studied in vitro using S9 liver preparation and had a t₁/² of 41.5 minutes with 2 metabolites (unidentified).³⁷ These results show that both, Substance P antagonist D and [D-Arg¹,D-Trp⁵,⁷,⁹,D-Leu¹⁰,Leu¹¹]Substance P-OH, are more stable than Substance P antagonist G. Also, collectively, these Substance P analogues are more stable than Substance P.

1.4.2.5 Phase I clinical trial of Substance P antagonist G

Substance P antagonist G is the only Substance P analogue to be tested in a Phase I clinical trial, which dates back to 2001.¹⁴² Since then, no further clinical trials on Substance P antagonist G or any Substance P analogues has been conducted. However, it is worth mentioning that this clinical trial was not intended to test the efficacy of Substance P antagonist G against SCLC. The study had two stages: stage 1 was designed to achieve safe plasma concentrations in humans to match with concentrations needed for anti-tumour activity, as observed from preclinical studies. Stage 2 was to test Substance P antagonist G activity against neuropeptide receptors. The activity against the receptors was evaluated by measuring the forearm blood flow (FBF), by venous occlusion plethysmography, in the presence of vasodilators before and during Substance P antagonist G administration.

The selected patients (24) in the trial were diagnosed with malignant tumours (renal, colon, NSCLC, SCLC, mesothelioma and others) in which the treatment received
Chapter 1

had failed. Patients recruited had at least 3 months of life expectancy, adequate hepatic, renal and cardiac functions and blood tests, and were aged ≥18 years. Substance P and bradykinin were the two neuropeptides selected for their known vasodilatory effect on the forearm.\textsuperscript{143-147}

For stage 1 (15 patients), the target plasma concentration of Substance P antagonist G needed was $C_{\text{max}} \geq 10 \ \mu\text{M}$ and was achieved \textit{via} IV infusion of an Substance P antagonist G dose of 300 mg/m\textsuperscript{2}. Substance P antagonist G doses of $>200 \ \text{mg/m}^2$ were safe, but resulted in facial flushing that started around 90 minutes of the 6 hours infusion time and lasted for $\sim 2$ hours after stopping infusion. Facial flushing was completely resolved within 24 hours. Substance P antagonist G doses of less than 200 mg/m\textsuperscript{2} had no side-effects. The first aim for the study was thus achieved; safe plasma concentration of Substance P antagonist G in humans equivalent to anti-tumour preclinical animal studies.

The FBF studies in stage 2 (9 patients) was performed by infusing Substance P antagonist G into the brachial artery of one of the arms at a dose of 400 mg/m\textsuperscript{2} to achieve Substance P antagonist G plasma concentration of $>20 \ \mu\text{M}$. Substance P and bradykinin caused a dose-dependent increase in FBF due to their vasodilatory affect. However, with the presence of Substance P antagonist G, the increase in FBF by Substance P and bradykinin was attenuated by up to 66% and 33%, respectively. Thus, the ability of Substance P antagonist G to antagonise the vasodilatory effect of Substance P and bradykinin was also achieved, showing that that Substance P antagonist G acts on different neuropeptide receptors. This is consistent with its description as a broad-spectrum neuropeptide antagonist.

On the other hand, the pharmacokinetic studies of Substance P antagonist G showed that it is rapidly excreted from the body when its infusion was stopped. Plasma concentrations $>10 \ \mu\text{M}$ were maintained only for $330 \pm 21$ minutes, but declined rapidly post-infusion. In terms of anti-tumour activity over a period of $8 \pm 2$ weeks, disease had progressed in 14 patients, remained stable in 3 patients and 7 patients
could not be evaluated. Although Substance P antagonist G showed inhibitory effect of Substance P and bradykinin induced vasodilation in humans at a safe concentration, the study suggested that this concentration may not be adequate for anti-tumour activity.

Concluding, Substance P antagonist G showed low cytotoxicity, with high IC\textsubscript{50} values (>10 µM) for SCLC cell lines. In addition, Substance P antagonist G was also prone to degradation. These limitations might be responsible for the clinical trial not proceeding to the next level. It was also shown that the metabolites of Substance P antagonist G and Substance P antagonist D did not maintain the inhibitory effect, when compared to their parent peptides, in inhibiting neuropeptides-stimulated growth of Swiss 3T3 cells. Hence, further optimisation of Substance P antagonist G / Substance P antagonist D or to design and develop novel potent and stable peptides is justifiable to improve efficacy.

1.5 Aims and objectives

1.5.1 Aims

The current treatment options for SCLC are limited and of low efficacy. This is due to the aggressive nature of the disease and its ability to relapse and become resistant to medications. Although there are several compounds being investigated for SCLC treatment, the development of neuropeptide antagonists, Substance P analogues, is of great importance. Substance P analogues give a better approach for SCLC treatment due to their broad-spectrum activity on multiple receptors rather than on a single target. Therefore, they may have better potential to overcome resistance.

The aforementioned Substance P analogues (Table 3) showed variable cytotoxic activity against SCLC. The general common features shared among them are the presence of amino acids in their D-isomers at the peptides' N-terminal and the presence of D-Tryptophan (D-Trp) and Leu residues. Also, some of the potent peptides have their N-terminal protected with either acylation or N-methylated amino acids. All of these modifications had already been shown to produce effective and
stable SP analogues against neurokinins. Moreover, the shorter hexapeptides (Substance P antagonist G and NY3460) and pentapeptides (NY3238 and NY3521), which are modifications of the C-terminal of Substance P peptide, were efficacious for the treatment of SCLC. Subsequently, these modifications and changes could be utilized for designing further Substance P analogues.

Some of the Substance P analogues had their stability assessed in plasma, liver and/or SCLC tumour homogenates. They were more stable than the parent Substance P peptide. However, their stability needs further improvement as they had short half-lives and relatively inactive metabolites. Another issue being observed was the hydrophobicity of some peptides due to the hydrophobic amino acids within their sequence. This hampered the use of higher concentrations using s.c. injection and even some i.p injections doses were not as efficacious as those of s.c. doses.

These issues should be considered when developing further analogues, with higher potency against SCLC, enhanced stability and solubility required. Therefore, the overall aim of this project was to design and synthesise further Substance P analogues and biologically evaluate their cytotoxicity against SCLC. The project intends to achieve potent and stable Substance P analogues for the treatment of SCLC.

The common strategies for the development of the previous analogues were to use different amino acids in their D-isomers, N-methylated amino acids and shortening of the peptides. One of this project’s aims is to introduce a novel strategy for developing short Substance P analogues, by applying modifications on the amino acids side chain. The indole ring of Trp lends itself as a site for modification. There are several natural and synthetic indole-containing compounds reported to have anti-tumour activity and some are still in clinical trials. The indole group of those compounds was modified either on its nitrogen atom or on the carbons of the cyclic system. Furthermore, a study was conducted to test the cytotoxic activity of modified L-Trp amino acids on cancer cell lines in which alkyl groups were added to indole.
In comparison to the unmodified L-Trp, the alkylated L-Trp amino acids were cytotoxic, but in the millimolar range. Thus, peptides with incorporated modified Trp amino acids within Substance P analogue sequence can be expected to enhance their cytotoxic activity against SCLC.

To enhance their stability, as in the previous analogues, the use of D-isomers of amino acids and protecting the N-terminal of the peptides sequences with N-methylated amino acid will be considered. Furthermore, it seems that the C-terminal rather than the N-terminal of the Substance P analogues is prone to degradation, as was observed with Substance P antagonist G and Substance P antagonist D. Substance P antagonist G also had another form of metabolism due to the oxidation of Met residue. It was also suggested that both peptides are being metabolised by carboxypeptidases, which have de-amidase and carboxypeptidase functions. Moreover, as Substance P antagonist D was more stable than Substance P antagonist G, the length of Substance P antagonist D might have altered the conformation of the peptide making its recognition to the enzyme differ from Substance P antagonist G. In addition, the lower activity of Substance P antagonist G and Substance P antagonist D metabolites, which were the results of C-terminal degradation, shows that the C-terminal is essential for peptides activity. Leading from this, the avoidance of using Met and applying modifications near the C-terminal of the peptides sequences might be an appropriate strategy to enhance both the stability and cytotoxic activity of the novel Substance P analogues.

For enhancing the solubility of the peptides, several approaches are possible. These include adding a charged amino acid, removing a hydrophobic amino acid and formulation with liposomes.

Knowing the cytotoxic activity of the hexapeptide NY3460 (DMePhe-DTrp-Phe-DTrp-Leuψ(CH₂NH)Leu-NH₂) on SCLC in vitro and in vivo, a similar and a shorter novel pentapeptide sequence will serve as the lead peptide to apply the
modifications. The lead peptide sequence of interest comprises DMePhe-DTrp-Phe-
DTrp-Leu-NH₂.

1.5.2 Objectives
To achieve the above aims, the following objectives were undertaken:

1. Synthesis of modified D-Trp amino acids precursors suitable for peptide
   synthesis with modifications at the indole nitrogen. Characterisation of the
   final products by proton (¹H) and carbon (¹³C) Nuclear Magnetic Resonance
   (NMR) spectroscopy and mass spectrometry (MS).
2. Design and synthesis of peptides by utilising solid and/or liquid phase
   procedures to incorporate the modified amino acids into sequences.
   Purification of peptides using Reversed Phase - High Performance Liquid
   Chromatography (RP-HPLC) and structure confirmation with MS and/or ¹H
   NMR spectroscopy.
3. Biological evaluation of peptides to measure their cytotoxic activity against
   classic and variant SCLC cell lines.
4. Studying the stability of the peptides in plasma and S9 liver fraction from
   mouse and preparing a liposomal formulation to address the main concerns
   regarding peptide delivery.
Chapter 2

Synthesis of Modified D-Tryptophan Derivatives
2. Synthesis of Modified D-Tryptophan Derivatives

2.1 Introduction

A first requirement was to make modified D-Trp derivatives with different substitutions on the indole nitrogen (N$^{\text{ind}}$), suitable for peptide synthesis. The selection of the D-Trp precursor to carry out the N$^{\text{ind}}$-modification reactions was based on the method that will be used for peptide synthesis.

Initially, it was planned that the peptide synthesis would be performed using Solid Phase Peptide Synthesis (SPPS), in which amino acids are coupled onto a fixed solid insoluble support (resin). SPPS was first introduced by Merrifield$^{151}$ and was selected for its well-known simplicity and speed in synthesising peptides. There are two strategies for peptide synthesis using the SPPS procedure. The first uses tert-butyloxy carbonyl (Boc)$^{152}$ protection chemistry and the second uses 9-fluorenylmethyloxycarbonyl (Fmoc)$^{153}$ protection chemistry. Boc and Fmoc represent the amine protecting groups of the amino acids used in the synthesis.

Depending on the reaction conditions needed to carry out the N$^{\text{ind}}$-modification and the stability of the amine protecting groups, either commercially available Boc protected D-Trp (Boc-D-Trp) or Fmoc protected D-Trp (Fmoc-D-Trp) (Figure 5) were used.

![Figure 5. Structures of Boc-D-Tryptophan (a) and Fmoc-D-Tryptophan (b)](image)

Although the amine protected N$^{\text{ind}}$-substituted D-Trp derivatives were compatible with SPPS procedures, only a few peptides were made using this method. The
syntheses of the other peptides were completed by Liquid Phase Peptide Synthesis (LPPS) to avoid inadequate precipitation of some peptides following cleavage from the resin in SPPS (discussed in Chapter 3). Boc and Fmoc N\textsuperscript{ind}-substituted D-Trp derivatives were also compatible with LPPS.

This chapter presents the detailed synthesis and characterisation of N\textsuperscript{ind}-substituted D-Trp derivatives.

2.2 Materials and Instrumentation

2.2.1 Materials

The Boc and Fmoc amine-protected D-Trp were obtained from Novabiochem. Thin Layer Chromatography (TLC) plates, TLC Silica gel 60 F\textsubscript{254} Aluminium sheets, were obtained from Merck Millipore. Silica gel (ZEOpREP 60/ 40-63 microns) was obtained from Apollo Scientific. Solvents and other chemicals were obtained from Sigma-Aldrich and Fisher Scientific.

2.2.2 Instrumentation

\textsuperscript{1}H and \textsuperscript{13}C NMR spectroscopy were performed using a Bruker Avance 400 MHz (\textsuperscript{1}H) and 75 MHz (\textsuperscript{13}C) spectrometer and spectra were generated using TOPSPIN 2.1 software. Chemical shifts (\(\delta\)) are quoted in parts per million (ppm) and referenced to residual solvent peak. Abbreviations used for splitting patterns are: s, singlet; br-s, broad singlet; d, doublet; dd, doublet of doublet; br-d, broad doublet; t, triplet; q, quartet; m, multiplet and br-m, broad multiplet. Electrospray Ionisation Mass Spectrometry (ESIMS) (Waters SQD-2 Single Quadrupole Mass Spectrometer attached to Acquity UHPLC) and Atmospheric Pressure Chemical Ionisation Mass Spectrometry (APCIMS) (Agilent Technologies 6120 Quadrupole LC/MS) were performed at the School of Chemistry, University of Manchester: molecular ion peaks are reported as mass/charge (\(m/z\)) ratios. Spots on TLC silica plates were visualised using UV Mineralight lamp (254/365) UVGL-58. Solvents were evaporated on a Buchi rotary evaporator equipped with a Buchi Vacuum Pump V-
Chapter 2

700 and Buchi heating bath Rotavapor R-3. Lyophilisation was performed using a Christ freeze-dryer.

2.3 Results and Discussion

2.3.1 Synthesis of $N^\text{ind}$-substituted derivatives of Boc-D-Tryptophan

Boc-D-Trp was reacted with 2 molar equivalents of potassium tert-butoxide ($t$-BuOK) to de-protonate the $N^\text{ind}$ and the carboxylic acid. Acting as nucleophiles, the de-protonated species reacted with the appropriate alkyl, propargyl or benzyl halide (R-X) (2 molar equivalents) forming the $N^\text{ind}$-substituted Boc-D-Trp derivatives in their ester form; Boc-D-Trp(N-R)-O-R (Figure 6). Boc-D-Trp was used since the Boc group is stable under the basic conditions, in contrast to the Fmoc group.

\[
\begin{align*}
\text{Boc-D-Trp(N-methyl)-O-methyl (1)} & : R=\text{Me} \\
\text{Boc-D-Trp(N-ethyl)-O-ethyl (2)} & : R=\text{Et} \\
\text{Boc-D-Trp(N-propyl)-O-propyl (3)} & : R=\text{Pr} \\
\text{Boc-D-Trp(N-butyl)-O-butyl (4)} & : R=\text{Bu} \\
\text{Boc-D-Trp(N-pentyl)-O-pentyl (5)} & : R=\text{Pe} \\
\text{Boc-D-Trp(N-propargyl)-O-propargyl (6)} & : R=\text{CH}_2\text{CCH} \\
\text{Boc-D-Trp(N-benzyl)-O-benzyl (7)} & : R=\text{CH}_2\text{Ph}
\end{align*}
\]

Figure 6. Structures of the $N^\text{ind}$-substituted Boc-D-Tryptophan ester derivatives

The Boc-D-Trp(N-R)-O-R derivatives were then reacted with lithium hydroxide (LiOH) to hydrolyse the ester and then acidified with hydrochloric acid (HCl) to protonate the carboxylate ion. This gave the $N^\text{ind}$-substituted Boc-D-Trp derivatives in their free carboxylic acid form; Boc-D-Trp(N-R)-OH (Figure 7). The concentration of HCl used (1 M) was suitable to keep the Boc group intact although it is acid labile. The Boc group is usually cleaved using 50% v/v trifluoroacetic acid.
(TFA)/dichloromethane (DCM), as for SPPS Boc protection chemistry described in Chapter 3. The mechanisms for the reactions are shown in Scheme 1.

![Scheme 1](image)

**Figure 7.** Structures of the N^ind-substituted Boc-D-Tryptophan derivatives in their neutral carboxylic acid form
2.3.1.1 Characterisation of Boc-D-Trp(N-methyl)-O-methyl (1) and Boc-D-Trp(N-methyl)-OH (8) by $^1$H NMR spectroscopy

Representative $^1$H NMR spectra for the modification of N\textsuperscript{ind} of Boc-D-Trp are provided in this section. The synthesis of compound 8 with the N\textsuperscript{ind}-methyl group was chosen as an example.

The $^1$H NMR spectrum for the un-substituted starting material Boc-D-Trp included peaks for the N\textsuperscript{ind}-H (s, 1H, Ind-1-NH) at $\delta$: 8.09 and the tert-butyl group of Boc (t-Bu) at $\delta$: 1.43 (s, 9H, t-Bu) (spectrum not shown). However, the -COOH group could not be observed.

The $^1$H NMR (400 MHz, CDCl\textsubscript{3}) spectrum (Figure 8) for 1 showed the peak for the t-Bu group at $\delta$: 1.44 (s, 9H, t-Bu), the disappearance of the Ind-1-NH peak and the appearance of the peaks for the two added methyl groups at $\delta$: 3.75 (s, 3H, N-CH\textsubscript{3} on Ind) and 3.69 (s, 3H, OCH\textsubscript{3} ester).
The $^1$H NMR (400 MHz, CDCl$_3$) spectrum (Figure 9) for carboxylic acid 8 was similar to that for 1, except that the peak for the methyl ester group was absent. The peaks include $\delta$: 3.74 (s, 3H, N-CH$_3$), and 1.43 (s, 9H, t-Bu). However, the -COOH group could not be observed.
2.3.2 Synthesis of N\textsuperscript{ind}-substituted derivatives of Fmoc-D-Tryptophan

Fmoc-D-Trp was used for the addition of the tert-prenyl group on its N\textsuperscript{ind}. It was used since Fmoc chemistry is safer and simpler than Boc chemistry for peptide synthesis (discussed in Chapter 3).

Fmoc-D-Trp was reacted with 2 molar equivalents of N,N'-dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS) to form the NHS ester of Fmoc-D-Trp; Fmoc-D-Trp-O-Su (15) (Figure 10). This was achieved by the carboxyl activation of Fmoc-D-Trp via reaction with DCC to form the unstable O-acylurea intermediate, which in turn reacts with NHS to form the stable NHS ester and the insoluble dicyclohexylurea (DCU). Scheme 2 represents the mechanism for the general synthesis of NHS amino acids esters.\textsuperscript{154,155}

![Figure 10. Structure of the NHS ester of Fmoc-D-Tryptophan (15)](image)
Scheme 2. Mechanism for the synthesis of NHS amino acid esters. $R = \text{CHR}_1\text{-NH}_2$, $R' = \text{cyclohexyl}$, $R_1$ = amino acid side chain.

The addition of the tert-prenyl group onto the $N^{\text{ind}}$ of 15 was achieved using the procedure described by Luzung and co-workers. Compound 15 was reacted with 2 molar equivalents of the oxidising agents, copper (II) acetate [Cu(OAc)$_2$] and silver trifluoroacetate [AgTFA], 40 mol% (a total of 4 equal additions) of the catalyst palladium (II) acetate [Pd(OAc)$_2$], and 30 molar equivalents of 2-methyl-2-buten to form Fmoc-D-Trp(N-tert-prenyl)-O-Su (16) (Figure 11).

Figure 11. Structure of the $N^{\text{ind}}$-tert-prenyl derivative of the NHS ester of Fmoc-D-Tryptophan (16)
The mechanism for the formation of (16) is through the C-H activation of 2-methyl-2-butene with Pd (II) as proposed by Luzung and co-workers\textsuperscript{156} to form the palladated olefin intermediate (Figure 12), which reacts with the indole group.

![Figure 12](image12.png)

**Figure 12.** Palladated 2-methyl-2-butene (redrawn from Luzung et al.\textsuperscript{156})

Luzung and co-workers proposed two ways in which the activated olefin reacts with the indole group, involving either the coordination of the indole nitrogen atom (N-1) (Figure 13 a) or the coordination of the indole carbon atom at position three (C-3) (Figure 13 b) to Pd.\textsuperscript{156} Throughout the reaction with the indole group, Pd (II) becomes reduced to Pd (0) and oxidised back to Pd (II) with the aid of the oxidising agents present Ag (I) and Cu (II).

![Figure 13](image13.png)

**Figure 13.** Olefin reaction modes with the indole group (redrawn from Luzung et al.\textsuperscript{156})

Finally, 16 was treated with sodium carbonate (Na\textsubscript{2}CO\textsubscript{3}) to hydrolyse the succinyl ester group and then acidified with HCl to form the carboxylic acid to give Fmoc-D-Trp(N-\textit{tert}-prenyl)-OH (17) (Figure 14). Although the Fmoc group is base labile, it remained intact under the mild basic conditions with the use of Na\textsubscript{2}CO\textsubscript{3}. 

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\textsuperscript{156} Luzung et al.
2.3.2.1 Characterisation of Fmoc-D-Trp-O-Su (15), Fmoc-D-Trp(N-tert-prenyl)-O-Su (16) and Fmoc-D-Trp(N-tert-prenyl)-OH (17) by $^1$H NMR spectroscopy

The $^1$H NMR spectrum for the un-substituted starting material Fmoc-D-Trp included peaks for -COOH at $\delta$: 12.75 (br-s, 1H, -COOH) and for Ind-1-NH at $\delta$: 10.87 (s, 1H, Ind-1-NH) (spectrum not shown). It should be noted that most of the peaks for the Fmoc group are overlapping with those of the indole ring, making the $^1$H NMR spectrum very complex in the aromatic region.

The $^1$H NMR (400 MHz, DMSO-d$_6$) spectrum (Figure 15) for 15 showed a peak for the Ind-1-NH at $\delta$: 10.96 (s, 1H, Ind-1-NH), the disappearance of the -COOH peak and the appearance of the peak for the succinyl group at $\delta$: 2.84 (s, 4H, 2 x CH$_2$ of Su).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure14.png}
\caption{Structure of the N$^{\text{Ind}}$-tert-prenyl derivative of Fmoc-D-Tryptophan in its neutral carboxylic acid form (17)}
\end{figure}
Figure 15. $^1$H NMR (400 MHz, DMSO-$d_6$) spectrum for Fmoc-D-Trp-O-Su (15).

The $^1$H NMR (400 MHz, DMSO-$d_6$) spectrum (Figure 16) for Fmoc-D-Trp(N-tert-prenyl)-O-Su 16 showed the peak for the succinyl group, the disappearance of the Ind-1-NH peak and the appearance of the peaks for the added tert-prenyl group. The peaks are presented as $\delta$: 6.07 (dd, 1H, $J = 17.6, 10.8$ Hz, N-C(CH$_3$)$_2$CHCH$_2$), 5.16 (d, 1H, $J = 10.8$ Hz, N-C(CH$_3$)$_2$CHCH$_2$), 5.10 (d, 1H, $J = 17.2$ Hz, N-C(CH$_3$)$_2$CHCH$_2$), 2.83 (s, 4H, 2 x CH$_2$ of Su), 1.66 (s, 3H, N-C(CH$_3$)$_2$CHCH$_2$) and 1.65 (s, 3H, N-C(CH$_3$)$_2$CHCH$_2$).
Figure 16. $^1$H NMR (400 MHz, DMSO-d$_6$) spectrum for Fmoc-D-Trp(N-tert-prenyl)-O-Su (16).

The $^1$H NMR (400 MHz, DMSO-d$_6$) spectrum (Figure 17) for Fmoc-D-Trp(N-tert-prenyl)-OH 17 was similar to that for 16, except that the peak for the succinyl group was absent. The peaks are presented as $\delta$: 6.07 (dd, 1H, J = 17.6, 10.8 Hz, N-C(CH$_3$)$_2$CHCH$_2$), 5.16 (d, 1H, J = 10.8 Hz, N-C(CH$_3$)$_2$CHCH$_2$), 5.10 (d, 1H, J = 17.2 Hz, N-C(CH$_3$)$_2$CHCH$_2$), 1.65 (s, 3H, N-C(CH$_3$)$_2$CHCH$_2$) and 1.64 (s, 3H, N-C(CH$_3$)$_2$CHCH$_2$). However, the -COOH group could not be observed.
Figure 17. $^1$H NMR (400 MHz, DMSO-d$_6$) spectrum for Fmoc-D-Trp(N-tert-prenyl)-OH (17).

2.3.3 Synthesis of N$_{\text{ind}}$-butyl D-Tryptophan

The N$_{\text{ind}}$-butyl derivative of D-Trp (D-Trp(N-butyl)-OH) (18) (Figure 18) was synthesised in order to test its efficacy on SCLC cells as a single N$_{\text{ind}}$-modified free amino acid. The results obtained from cell viability assays performed using peptides that have D-Trp(N-butyl) in their sequences are discussed in Chapter 4. Boc-D-Trp(N-butyl)-OH (11) was treated with 50% v/v TFA/DCM for 1 hour to cleave the Boc group to give (18).

Figure 18. Structure of the N$_{\text{ind}}$-butyl derivative of D-Tryptophan (18)
2.4 Experimental

2.4.1 Boc-D-Trp-OH

\[
\begin{align*}
\text{H NMR (400 MHz, CDCl}_3\text{)) } & : 8.09\text{ (s, 1H, Ind-1-NH)},
7.60\text{ (d, 1H, J 7.6 Hz, Ar-H),}
7.36\text{ (d, 1H, J 7.6 Hz, Ar-H),}
7.21\text{ (t, 1H, J 7.2 Hz, Ar-H),}
7.12\text{ (t, 1H, J 7.4 Hz, Ar-H),}
7.02\text{ (s, 1H, Ind-2-H),}
5.04\text{ (br-d, 1H, J 6.8 Hz, NHBoc),}
4.60 - 4.70\text{ (br-m, 1H, CH),}
3.38 - 3.29\text{ (m, 2H, CH}_2\text{),}
1.43\text{ (s, 9H, t-Bu).}
\end{align*}
\]

2.4.2 Boc-D-Trp(N-methyl)-O-methyl (1)

\[
\begin{align*}
\text{A solution of t-BuOK (0.74 g, 6.58 mmol) in dry tetrahydrofuran (THF) (10 ml) was added dropwise to a solution of Boc-D-Trp (1.0 g, 3.29 mmol) in dry N,N-dimethylformamide (DMF) (10 ml) under N}_2\text{ atmosphere at 0 }^\circ\text{C. The mixture was stirred for 20 minutes at room temperature (RT). Iodomethane (410 µl, 6.58 mmol) was then added to the above mixture under N}_2\text{ atmosphere at 0 }^\circ\text{C. The mixture was stirred at RT for 8 hours. Reaction completion was checked by TLC eluting with ethyl acetate/hexane (1:1). Citric acid aqueous solution (30% w/v) (200 ml) was then added and the mixture was extracted with ethyl acetate (4 x 50 ml). The organic layers were combined, dried over anhydrous sodium sulphate (Na}_2\text{SO}_4\text{), and concentrated under reduced pressure. The crude product was purified by column chromatography (ethyl acetate/hexane, 1:4), TLC } R_f\text{ 0.25. Fractions were collected.}
\end{align*}
\]
and concentrated under reduced pressure to give 0.75 g (68.7%) of 1 as a yellow oil.

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$: 7.54 (d, 1H, J 7.6 Hz, Ar-H), 7.29 (d, 1H, J 8.0 Hz, Ar-H), 7.23 (t, 1H, J 7.6 Hz, Ar-H), 7.11 (t, 1H, J 7.4 Hz, Ar-H), 6.86 (s, 1H, Ind-2-H), 5.07 (br-d, 1H, J 7.6 Hz, NHBOc), 4.64 (dd, 1H, J 13.2, 5.2 Hz, CH), 3.75 (s, 3H, N-CH$_3$ on Ind), 3.69 (s, 3H, OCH$_3$ ester), 3.33-3.24 (m, 2H, CH$_2$), 1.44 (s, 9H, t-Bu).

$^{13}$C NMR (75 MHz, CDCl$_3$, assignments made using DEPT-135) $\delta$: 172.9 (C, C12), 155.4 (C, C13), 137.1 (C, Ar-C), 128.3 (C, Ar-C), 127.6 (CH, Ar-C), 121.9 (CH, Ar-C), 119.2 (CH, Ar-C), 119.0 (CH, Ar-C), 109.4 (CH, Ar-C), 108.7 (C, Ar-C), 79.9 (C, C14), 54.4 (CH, C11), 52.3 (OCH$_3$ ester), 32.8 (N-CH$_3$ on Ind), 28.4 (3 x CH$_3$, C15, C16, C17), 28.0 (CH$_2$, C10). MS (ESI) $m/z$ [M+Na]$^+$ 355.2. Accurate mass calculated for C$_{18}$H$_{24}$N$_2$O$_4$Na: 356.1628. Found: MS (ESI) 356.1637, error 2.5 ppm.

### 2.4.3 Boc-D-Trp(N-ethyl)-O-ethyl (2)

The method of synthesis was similar to that described for 1, except that iodoethane (526 µl, 6.58 mmol) was used. A yellow oil (0.67 g, 56.6%) of 2 was isolated after column chromatography, TLC $R_f$ 0.37. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$: 7.56 (d, 1H, J 7.6 Hz, Ar-H), 7.31 (d, 1H, J 8.4 Hz, Ar-H), 7.21 (t, 1H, J 8.0 Hz, Ar-H), 7.10 (t, 1H, J 7.4 Hz, Ar-H), 6.93 (s, 1H, Ind-2-H), 5.07 (br-d, 1H, J 7.6 Hz, NHBOc), 4.62 (dd, 1H, J 13.6, 5.6 Hz, CH), 4.13 (q, 2H, J 7.1 Hz, CH$_2$CH$_3$), 4.13 (q, 2H, J 7.3 Hz, CH$_2$CH$_3$), 3.32-3.23 (m, 2H, CH$_2$), 1.44 (s, 9H, t-Bu), 1.44 (t, 3H, J 7.2 Hz, 3H of OCH$_2$CH$_3$ ester), 1.20 (t, 3H, J 7.2 Hz, N-CH$_2$CH$_2$ on Ind). $^{13}$C NMR (75 MHz, CDCl$_3$, assignments made using DEPT-135) $\delta$: 172.5 (C, C12), 155.4 (C, C13), 136.1 (C, Ar-C), 128.6 (C, Ar-C), 125.8 (CH, Ar-C), 121.7 (CH, Ar-C), 119.1 (2 x CH, Ar-C), 109.4 (CH, Ar-C), 109.0 (C, Ar-C), 79.8 (C, C14), 61.3 (CH$_2$, OCH$_2$CH$_3$ ester), 54.5 (CH, C11), 40.9 (CH$_2$, N-CH$_2$CH$_3$ on Ind), 28.5 (3 x CH$_3$, C15, C16,
C17), 28.2 (CH₂, C10), 15.5 (CH₃, N-CH₂CH₃ on Ind), 14.2 (CH₃, CH₂CH₃ on ester).


2.4.4 Boc-D-Trp(N-propyl)-O-propyl (3)

The method of synthesis was similar to that described for 1, except that 1-iodopropane (642 µl, 6.58 mmol) was used. A yellow oil (0.75 g, 58.8%) of 3 was isolated after column chromatography, TLC Rf 0.42. ¹H NMR (400 MHz, CDCl₃) δ:

7.56 (d, 1H, J 8.0 Hz, Ar-H), 7.30 (d, 1H, J 8.0 Hz, Ar-H), 7.20 (t, 1H, J 7.6 Hz, Ar-H), 7.10 (t, 1H, J 7.4 Hz, Ar-H), 6.91 (s, 1H, Ind-2-H), 5.07 (br-d, 1H, J 7.6 Hz, NHBoc), 4.63 (dd, 1H, J 13.2, 5.6 Hz, CH), 4.05-3.99 (m, 4H, CH₂CH₂CH₃ on Ind and ester), 3.30 (dd, 1H, J 14.2, 5.0 Hz, 10-CH₂A), 3.26 (dd, 1H, J 14.6, 5.0 Hz, 10-CH₂B), 1.84 (sextet, 2H, J 7.2 Hz, N-CH₂CH₂CH₃ on Ind), 1.59 (sextet, 2H, J 7.0 Hz, OCH₂CH₂CH₃ ester), 1.44 (s, 9H, t-Bu), 0.91 (t, 3H, J 7.4 Hz, OCH₂CH₂CH₂ ester), 0.86 (t, 3H, J 7.4 Hz, N-CH₂CH₂CH₂ ester).

¹³C NMR (75 MHz, CDCl₃, assignments made using DEPT-135) δ: 172.5 (C, C12), 155.4 (C, C13), 136.4 (C, Ar-C), 128.5 (C, Ar-C), 126.6 (CH, Ar-C), 121.7 (CH, Ar-C), 119.1 (2 x CH, Ar-C), 109.5 (CH, Ar-C), 108.8 (C, Ar-C), 79.8 (C, C14), 66.9 (CH₂, OCH₂CH₂CH₃ ester), 54.5 (CH, C11), 48.0 (CH₂, N-CH₂CH₂CH₃ on Ind), 28.4 (3 x CH₃, C15, C16, C17), 28.2 (CH₂, C10), 23.6 (CH₂, N-CH₂CH₂CH₃ on Ind), 22.0 (CH₂, OCH₂CH₂CH₃ ester), 11.6 (CH₃, N-CH₂CH₂CH₃ on Ind), 10.4 (CH₃, OCH₂CH₂CH₃ ester). MS (ESI) m/z [M+Na]⁺ 411.2. Accurate mass calculated for C₂₂H₃₂N₂O₄Na: 411.2254. Found: MS (ESI) 411.2248, error -1.5 ppm.
2.4.5 Boc-D-Trp(N-butyl)-O-butyl (4)

The method of synthesis was similar to that described for 1, except that 1-iodobutane (749 µl, 6.58 mmol) was used. A yellow oil (1.03 g, 75.3%) of 4 was isolated after column chromatography, TLC *R*$_f$ 0.58. $^1$H NMR (400 MHz, CDCl$_3$) δ: 7.55 (d, 1H, $J$ 7.6 Hz, Ar-H), 7.30 (d, 1H, $J$ 8.4 Hz, Ar-H), 7.20 (t, 1H, $J$ 7.6 Hz, Ar-H), 7.10 (t, 1H, $J$ 7.2 Hz, Ar-H), 6.90 (s, 1H, Ind-2-H), 5.07 (br-d, 1H, $J$ 8.0 Hz, NHBoc), 4.63 (dd, 1H, $J$ 13.6, 5.6 Hz, CH), 4.10-4.03 (m, 4H, CH$_2$CH$_2$CH$_2$CH$_3$ on Ind and ester), 3.32-3.23 (m, 2H, CH$_2$), 1.79 (pentet, 2H, $J$ 7.3 Hz, N-CH$_2$CH$_2$CH$_2$CH$_3$ on Ind), 1.53 (pentet, 2H, $J$ 7.2 Hz, OCH$_2$CH$_2$CH$_2$CH$_3$ ester), 1.44 (s, 9H, t-Bu), 1.30 (sextet, 4H, $J$ 7.5 Hz, CH$_2$CH$_2$CH$_2$CH$_3$ on Ind and ester), 0.94 (t, 3H, $J$ 7.4 Hz, OCH$_2$CH$_2$CH$_3$ ester), 0.89 (t, 3H, $J$ 7.4 Hz, N-CH$_2$CH$_2$CH$_2$CH$_3$ on Ind). $^{13}$C NMR (75 MHz, CDCl$_3$, assignments made using DEPT-135) δ: 172.5 (C, C$_{12}$), 155.4 (C, C$_{13}$), 136.4 (C, Ar-C), 128.5 (C, Ar-C), 126.6 (CH, Ar-C), 121.7 (CH, Ar-C), 119.1 (2 x CH, Ar-C), 109.5 (CH, Ar-C), 108.8 (C, Ar-C), 79.8 (C, C$_{14}$), 65.2 (CH$_2$, OCH$_2$CH$_2$CH$_2$CH$_3$ ester), 54.5 (CH, C$_{11}$), 46.1 (CH$_2$, N-CH$_2$CH$_2$CH$_2$CH$_3$ on Ind), 32.4 (CH$_2$, N-CH$_2$CH$_2$CH$_2$CH$_3$ on Ind), 30.6 (CH$_2$, OCH$_2$CH$_2$CH$_2$CH$_3$ ester), 28.5 (3 x CH$_3$, C$_{15}$, C$_{16}$, C$_{17}$), 28.2 (CH$_2$, C$_{10}$), 20.3 (CH$_2$, N-CH$_2$CH$_2$CH$_2$CH$_3$ on Ind), 19.1 (CH$_2$, OCH$_2$CH$_2$CH$_2$CH$_3$ ester), 13.80 (CH$_3$, N-CH$_2$CH$_2$CH$_2$CH$_3$ on Ind), 13.76 (CH$_3$, OCH$_2$CH$_2$CH$_2$CH$_3$ ester). MS (ESI) *m/z* [M+K]$^+$ 455.2. Accurate mass calculated C$_{24}$H$_{36}$N$_2$O$_4$K: 455.2307. Found: MS (ESI) 455.2308, error 0.2 ppm.
2.4.6 Boc-D-Trp(N-pentyl)-O-pentyl (5)

![Chemical Structure](image)

The method of synthesis was similar to that described for 1, except that 1-iodopentane (856 µl, 6.58 mmol) was used. A yellow oil (0.53 g, 36.3%) of 5 was isolated after column chromatography, TLC $R_f$ 0.65. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$: 7.54 (d, 1H, $J$ 8.0 Hz, Ar-H), 7.30 (d, 1H, $J$ 8.0 Hz, Ar-H), 7.19 (t, 1H, $J$ 7.6 Hz, Ar-H), 7.09 (t, 1H, $J$ 7.3 Hz, Ar-H), 6.90 (s, 1H, Ind-2-H), 5.06 (br-d, 1H, $J$ 8.0 Hz, NHBoc), 4.62 (dd, 1H, $J$ 13.6, 5.6 Hz, CH), 4.09-3.99 (m, 4H, C$_2$H$_2$CH$_2$CH$_2$CH$_3$ on Ind and ester), 3.32-3.21 (m, 2H, CH$_2$), 1.80 (pentet, 2H, $J$ 7.3 Hz, N-CH$_2$CH$_2$CH$_2$CH$_3$ on Ind), 1.55 (pentet, 2H, $J$ 7.1 Hz, OCH$_2$CH$_2$CH$_2$CH$_3$ ester), 1.43 (s, 9H, t-Bu), 1.37-1.21 (m, 8H, CH$_2$CH$_2$CH$_2$CH$_3$ on Ind and ester), 0.90-0.86 (m, 6H, CH$_2$CH$_2$CH$_2$CH$_3$ on Ind and ester). $^{13}$C NMR (75 MHz, CDCl$_3$, assignments made using DEPT-135) $\delta$: 172.6 (C, C12), 155.4 (C, C13), 136.3 (C, Ar-C), 128.5 (C, Ar-C), 126.5 (CH, Ar-C), 121.7 (CH, Ar-C), 119.1 (2 x CH, Ar-C), 109.5 (CH, Ar-C), 108.8 (C, Ar-C), 79.8 (C, C14), 65.6 (CH$_2$, OCH$_2$CH$_2$CH$_2$CH$_3$ ester), 54.5 (CH, C11), 46.4 (CH$_2$, N-CH$_2$CH$_2$CH$_2$CH$_3$ on Ind), 30.1 (CH$_2$, N-CH$_2$CH$_2$CH$_2$CH$_3$ on Ind), 29.3 (CH$_2$, N-CH$_2$CH$_2$CH$_2$CH$_3$ on Ind), 28.5 (CH$_2$, OCH$_2$CH$_2$CH$_2$CH$_3$ ester), 28.3 (3 x CH$_3$, C15, C16, C17), 28.2 (CH$_2$, C10), 28.1 (CH$_2$, OCH$_2$CH$_2$CH$_2$CH$_3$ ester), 22.5 (CH$_2$, N-CH$_2$CH$_2$CH$_2$CH$_3$ on Ind), 22.4 (CH$_2$, OCH$_2$CH$_2$CH$_2$CH$_3$ ester), 14.07 (CH$_3$, N-CH$_2$CH$_2$CH$_2$CH$_3$ on Ind), 14.04 (CH$_3$, OCH$_2$CH$_2$CH$_2$CH$_3$ ester). MS (ESI) $m/z$ [M+H]$^+$ 445.3. Accurate mass calculated for C$_{26}$H$_{40}$N$_2$O$_4$: 445.3061. Found: MS (ESI) 445.3058, error -0.7 ppm.
2.4.7 Boc-D-Trp(N-propargyl)-O-propargyl (6)

The method of synthesis was similar to that described for 1, except that propargyl bromide (709 µl, 6.58 mmol) was used. A yellow oil (0.60 g, 48%) of 6 was isolated after column chromatography, TLC Rf 0.29. ¹H NMR (400 MHz, CDCl₃) δ: 7.57 (d, 1H, J 7.6 Hz, Ar-H), 7.37 (d, 1H, J 8.4 Hz, Ar-H), 7.25 (t, 1H, J 7.8 Hz, Ar-H), 7.15 (t, 1H, J 7.2 Hz, Ar-H), 7.06 (s, 1H, Ind-2-H), 5.05 (br-d, 1H, J 8.0 Hz, NHBoc), 4.83 (br-d, 2H, 4J 2.8 Hz, N-CH₂CCH on Ind), 4.76-4.62 (m, 3H, 1H of CH and 2H of OCH₂CCH ester), 3.36-3.27 (m, 2H, CH₂), 2.50 (t, 1H, 4J 2.4 Hz, OCH₂CCH ester), 2.40 (t, 1H, 4J 2.6 Hz, N-CH₂CCH on Ind), 1.43 (s, 9H, t-Bu). ¹³C NMR (75 MHz, CDCl₃, assignments made using DEPT-135) δ: 171.6 (C, C12), 155.3 (C, C13), 136.2 (2 x C, Ar-C), 126.2 (CH, Ar-C), 122.4 (CH, Ar-C), 120.0 (CH, Ar-C), 119.3 (CH, Ar-C), 109.7 (C, Ar-C), 109.5 (CH, Ar-C), 80.1 (C, C14), 77.8 (C, N-CH₂CCH on Ind), 77.4 (C, OCH₂CCH ester), 75.5 (CH, OCH₂CCH ester), 73.8 (CH, N-CH₂CCH on Ind), 54.4 (CH, C11), 52.8 (CH₂, OCH₂CCH ester), 35.9 (CH₂, N-CH₂CCH on Ind), 28.5 (3 x CH₃, C15, C16, C17), 27.9 (CH₂, C10). MS (ESI) m/z [M+K]+ 419.1. Accurate mass calculated for C₂₂H₂₄N₂O₄K: 419.1368. Found: MS (ESI) 419.1378, error 2.4 ppm.
2.4.8 Boc-D-Trp(N-benzyl)-O-benzyl (7)

The method of synthesis was similar to that described for 1, except that benzyl bromide (783 µl, 6.58 mmol) was used. A yellow oil (1.08 g, 67.8%) of 7 was isolated after column chromatography, TLC $R_f$ 0.47. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$: 7.59 (d, 1H, $J$ 8.0 Hz, Ar-H), 7.32-7.22 (m, 9H, Ar-H), 7.19 (t, 1H, $J$ 7.0 Hz, Ar-H), 7.13 (t, 1H, $J$ 7.2 Hz, Ar-H), 7.05 (br-d, 2H, $J$ 7.2 Hz, Ar-H), 6.69 (s, 1H, Ind-2-H), 5.20 (br-s, 2H, 2H of N-CH$_2$Ph on Ind), 5.13 (br-d, 1H, $J$ 7.6 Hz, NHBoc), 5.11 (d, 1H, $J$ 12.4 Hz, OCH$_2$Ph ester), 5.04 (d, 1H, $J$ 12.4 Hz, OCH$_2$Ph ester), 4.73 (dd, 1H, $J$ 13.0, 5.0 Hz, CH), 3.35 (dd, 1H, $J$ 14.6, 5.0 Hz, 10-CH$_A$), 3.30 (dd, 1H, $J$ 14.2, 4.6 Hz, 10-CH$_B$), 1.45 (s, 9H, t-Bu). $^{13}$C NMR (75 MHz, CDCl$_3$, assignments made using DEPT-135) $\delta$: 172.1 (C, C12), 155.3 (C, C13), 137.6 (C, Ar-C on Ind), 136.7 (C, Ar-C), 135.5 (C, Ar-C ester), 128.8 (2 x CH, Ar-C on Ind), 128.6 (2 x CH, Ar-C on Ind), 128.5 (C, Ar-C), 128.4 (2 x CH, Ar-C on Ind and ester), 127.7 (2 x CH, Ar-C on Ind), 127.1 (2 x CH, Ar-C ester), 126.8 (CH, Ar-C), 122.0 (CH, Ar-C), 119.5 (CH, Ar-C), 119.2 (CH, Ar-C), 109.8 (CH, Ar-C), 109.4 (C, Ar-C), 79.8 (C, C14), 67.1 (CH$_2$, OCH$_2$Ph ester), 54.6 (CH, C11), 50.0 (CH$_2$, N-CH$_2$Ph on Ind), 28.4 (3 x CH$_3$, C15, C16, C17), 28.1 (CH$_2$, C10). MS (ESI) $m/z$ [M+K]$^+$ 523.2. Accurate mass calculated for C$_{30}$H$_{32}$N$_2$O$_4$K: 523.1994. Found: MS (ESI) 523.2017, error 4.4 ppm.
2.4.9 Boc-D-Trp(N-methyl)-OH (8)

An aqueous solution of 1 M LiOH (25 ml) was added to 0.7 g of 1 dissolved in THF (25 ml). The reaction was monitored by TLC, eluting with ethyl acetate/hexane (1:1). After completion of hydrolysis, the reaction was acidified by an aqueous solution of 1 M HCl and the mixture was extracted with ethyl acetate (3 x 50 ml). The organic layers were combined, washed with deionised water (H₂O) (2 x 20 ml), dried over Na₂SO₄, concentrated under reduced pressure, and lyophilised to give 0.60 g (89.5%) of 8 as a white solid. \(^1\)H NMR (400 MHz, CDCl₃) δ: 7.59 (d, 1H, J 8.0 Hz, Ar-H), 7.29 (d, 1H, J 8.0 Hz, Ar-H), 7.23 (t, 1H, J 7.4 Hz, Ar-H), 7.11 (t, 1H, J 7.4 Hz, Ar-H), 6.90 (s, 1H, Ind-2-H), 5.03 (br-d, 1H, J 7.2 Hz, NHBoc), 4.70-4.60 (br-m, 1H, CH), 3.74 (s, 3H, N-CH₃), 3.38-3.26 (m, 2H, CH₂), 1.43 (s, 9H, t-Bu). \(^{13}\)C NMR (75 MHz, CDCl₃, assignments made using DEPT-135) δ: 176.6 (C, C12), 155.8 (C, C13), 137.1 (C, Ar-C), 128.4 (C, Ar-C), 127.8 (CH, Ar-C), 122.0 (CH, Ar-C), 119.4 (CH, Ar-C), 119.0 (CH, Ar-C), 109.4 (CH, Ar-C), 108.5 (C, Ar-C), 80.4 (C, C14), 54.4 (CH, C11), 32.8 (CH₃, N-CH₃), 28.4 (3 x CH₃, C15, C16, C17), 27.6 (CH₂, C10). MS (ESI) m/z [M+Na]+ 341.1. Accurate mass calculated for C₁₇H₂₂N₂O₄Na: 341.1472. Found: MS (ESI) 341.1493, error 6.2 ppm.
2.4.10 Boc-D-Trp(N-ethyl)-OH (9)

The method of synthesis was similar to that described for 8; 1 M LiOH was added to 0.6 g of 2 to give 0.51 g (92.2%) of 9 as a white solid. $^1$H NMR (400 MHz, CDCl$_3$) δ: 7.59 (d, 1H, $J$ 7.6 Hz, Ar-H), 7.32 (d, 1H, $J$ 8.4 Hz, Ar-H), 7.21 (t, 1H, $J$ 7.6 Hz, Ar-H), 7.10 (t, 1H, $J$ 7.4 Hz, Ar-H), 6.98 (s, 1H, Ind-2-H), 5.02 (br-d, 1H, $J$ 7.2 Hz, NHBoc), 4.70-4.60 (br-m, 1H, CH), 4.12 (q, 2H, $J$ 6.8 Hz, N-C$_2$H$_3$CH$_3$), 3.36 (dd, 1H, $J$ 14.4, 5.2 Hz, 10-CH$_A$), 3.29 (dd, 1H, $J$ 14.8, 5.2 Hz, 10-CH$_B$), 1.43 (s, 9H, t-Bu), 1.26 (br, 3H, N-CH$_2$C$_3$H$_7$). $^{13}$C NMR (75 MHz, CDCl$_3$, assignments made using DEPT-135) δ: 176.6 (C, C12), 155.9 (C, C13), 136.1 (C, Ar-C), 128.5 (C, Ar-C), 126.1 (CH, Ar-C), 121.8 (CH, Ar-C), 119.3 (CH, Ar-C), 119.1 (CH, Ar-C), 109.5 (CH, Ar-C), 108.6 (C, Ar-C), 80.4 (C, C14), 54.4 (CH, C11), 41.0 (CH$_2$, N-CH$_2$CH$_3$), 28.4 (3 x CH$_3$, C15, C16, C17), 27.7 (CH$_2$, C10), 15.5 (CH$_3$, N-CH$_2$CH$_3$).

MS (ESI) $m/z$ [M+K]$^+$ 371.1. Accurate mass calculated for C$_{18}$H$_{24}$N$_2$O$_4$K: 371.1368. Found: MS (ESI) 371.1387, error 5.1 ppm.

2.4.11 Boc-D-Trp(N-propyl)-OH (10)

The method of synthesis was similar to that described for 8; 1 M LiOH was added to 0.7 g of 3 to give 0.56 g (89.7%) of 10 as a white solid. $^1$H NMR (400 MHz, CDCl$_3$) δ: 7.59 (d, 1H, $J$ 7.6 Hz, Ar-H), 7.31 (d, 1H, $J$ 8.0 Hz, Ar-H), 7.20 (t, 1H, $J$ 7.4 Hz,
Ar-H), 7.10 (t, 1H, J 7.4 Hz, Ar-H), 6.96 (s, 1H, Ind-2-H), 5.02 (br-d, 1H, J 7.6 Hz, NHBoc), 4.70-4.60 (br-m, 1H, CH), 4.03 (t, 2H, J 6.8 Hz, N-CH₂CH₂CH₃), 3.36 (dd, 1H, J 14.6, 5.0 Hz, 10-CH₃), 3.28 (dd, 1H, J 14.8, 5.6 Hz, 10-CH₂), 1.82 (sextet, 2H, J 7.1 Hz, N-CH₂CH₂CH₃), 1.43 (s, 9H, t-Bu), 0.89 (t, 3H, J 7.4 Hz, N-CH₂CH₂CH₃).

13C NMR (75 MHz, CDCl₃, assignments made using DEPT-135) δ: 176.9 (C, C12), 155.8 (C, C13), 136.4 (C, Ar-C), 128.4 (C, Ar-C), 127.0 (CH, Ar-C), 121.8 (CH, Ar-C), 119.3 (CH, Ar-C), 119.0 (CH, Ar-C), 109.6 (CH, Ar-C), 108.4 (C, Ar-C), 80.3 (C, C14), 54.4 (CH, C11), 48.1 (CH₂, N-CH₂CH₂CH₃), 28.4 (3 x CH₃, C15, C16, C17), 27.7 (CH₂, C10), 23.6 (CH₂, N-CH₂CH₂CH₃), 11.6 (CH₃, N-CH₂CH₂CH₃). MS (ESI) m/z [M+H]+ 347.2. Accurate mass calculated for C₁₉H₂₆N₂O₄H: 347.1965. Found: MS (ESI) 347.1979, error 4.0 ppm.

2.4.12 Boc-D-Trp(N-buty1)-OH (11)

The method of synthesis was similar to that described for 8; 1 M LiOH was added to 0.95 g of 4 to give 0.73 g (88.8%) of 11 as a white solid. 1H NMR (400 MHz, CDCl₃) δ: 7.59 (d, 1H, J 7.6 Hz, Ar-H), 7.31 (d, 1H, J 8.4 Hz, Ar-H), 7.20 (t, 1H, J 7.4 Hz, Ar-H), 7.10 (t, 1H, J 7.4 Hz, Ar-H), 6.96 (s, 1H, Ind-2-H), 5.02 (br-d, 1H, J 7.6 Hz, NHBoc), 4.70-4.60 (br-m, 1H, CH), 4.06 (t, 2H, J 6.6 Hz, N-CH₂CH₂CH₂CH₃), 3.36 (dd, 1H, J 14.8, 5.2 Hz, 10-CH₃), 3.28 (dd, 1H, J 14.8, 5.6 Hz, 10-CH₂), 1.78 (pentet, 2H, J 7.3 Hz, N-CH₂CH₂CH₂CH₃), 1.43 (s, 9H, t-Bu), 1.35-1.23 (m, 2H, N-CH₂CH₂CH₂CH₃), 0.92 (t, 3H, J 7.4 Hz, N-CH₂CH₂CH₂CH₃).

13C NMR (75 MHz, CDCl₃, assignments made using DEPT-135) δ: 176.9 (C, C12), 155.8 (C, C13), 136.4 (C, Ar-C), 128.4 (C, Ar-C), 126.9 (CH, Ar-C), 121.8 (CH, Ar-C), 119.2 (CH, Ar-C), 119.0 (CH, Ar-C), 109.6 (CH, Ar-C), 108.4 (C, Ar-C), 80.3 (C, C14), 54.4 (CH, C11), 46.1 (CH₂, N-CH₂CH₂CH₂CH₃), 32.4 (CH₂, N-CH₂CH₂CH₂CH₃), 28.4 (3 x CH₃, C15, C16, C17), 27.7 (CH₂, C10), 23.6 (CH₂, N-CH₂CH₂CH₃), 11.6 (CH₃, N-CH₂CH₂CH₃).
CH₂CH₂CH₂CH₃), 28.4 (3 x CH₃, C15, C16, C17), 27.7 (CH₂, C10), 20.3 (CH₂, N-CH₂CH₂CH₂CH₃), 13.8 (CH₃, N-CH₂CH₂CH₂CH₃). MS (ESI) m/z [M-H]⁻ 359.2. Accurate mass calculated for C₂₀H₂₈N₂O₄-H: 359.1976. Found: MS (ESI) 359.1965, error -3.1 ppm.

2.4.13 Boc-D-Trp(N-pentyl)-OH (12)

The method of synthesis was similar to that described for 8; 1 M LiOH was added to 0.45 g of 5 to give 0.31 g (81.8%) of 12 as a white solid. ¹H NMR (400 MHz, CDCl₃) δ: 7.59 (d, 1H, J 7.6 Hz, Ar-H), 7.31 (d, 1H, J 8.0 Hz, Ar-H), 7.20 (t, 1H, J 7.4 Hz, Ar-H), 7.09 (t, 1H, J 7.4 Hz, Ar-H), 6.96 (s, 1H, Ind-2-H), 5.01 (br-d, 1H, J 7.6 Hz, NHBoc), 4.70-4.60 (br-m, 1H, CH), 4.06 (t, 2H, J 7.0 Hz, N-CH₂CH₂CH₂CH₂CH₃), 3.35 (dd, 1H, J 14.8, 5.2 Hz, 10-CH₂), 3.28 (dd, 1H, J 14.8, 5.6 Hz, 10-CH₂), 1.79 (pentet, 2H, J 7.2 Hz, N-CH₂CH₂CH₂CH₂CH₃), 1.42 (s, 9H, t-Bu), 0.87 (t, 3H, J 7.0 Hz, N-CH₂CH₂CH₂CH₂CH₃). ¹³C NMR (75 MHz, CDCl₃, assignments made using DEPT-135) δ: 176.4 (C, C12), 155.8 (C, C13), 136.4 (C, Ar-C), 128.4 (C, Ar-C), 126.9 (CH, Ar-C), 121.8 (CH, Ar-C), 119.3 (CH, Ar-C), 119.0 (CH, Ar-C), 109.6 (CH, Ar-C), 108.4 (C, Ar-C), 80.4 (C, C14), 54.4 (CH, C11), 46.5 (CH₂, N-CH₂CH₂CH₂CH₂CH₃), 30.0 (CH₂, N-CH₂CH₂CH₂CH₂CH₃), 29.3 (CH₂, N-CH₂CH₂CH₂CH₂CH₃), 28.4 (3 x CH₃, C15, C16, C17), 27.7 (CH₂, C10), 22.4 (CH₂, N-CH₂CH₂CH₂CH₂CH₃), 14.1 (CH₃, N-CH₂CH₂CH₂CH₂CH₃). MS (ESI) m/z [M+H]⁺ 375.2. Accurate mass calculated for C₂₁H₃₀N₂O₄-H: 375.2278. Found: MS (APCI) 375.2277, error -0.3 ppm.
2.4.14 Boc-D-Trp(N-propargyl)-OH (13)

The method of synthesis was similar to that described for 8; 1 M LiOH was added to 0.55 g of 6 to give 0.45 g (90.9%) of 13 as a white solid. ^1H NMR (400 MHz, CDCl\textsubscript{3}) δ: 7.60 (d, 1H, J 8.0 Hz, Ar-H), 7.38 (d, 1H, J 8.4 Hz, Ar-H), 7.25 (t, 1H, J 7.4 Hz, Ar-H), 7.14 (t, 1H, J 7.4 Hz, Ar-H), 7.06 (s, 1H, Ind-2-H), 5.03 (br-d, 1H, J 7.6 Hz, NHBoc), 4.82 (br-s, 2H, N-CH\textsubscript{2}CH), 4.70-4.60 (br-m, 1H, CH), 3.36 (dd, 1H, J 14.6, 5.0 Hz, 10-CH\textsubscript{A}), 3.29 (dd, 1H, J 15.0, 5.4 Hz, 10-CH\textsubscript{B}), 2.39 (br-s, 1H, N-CH\textsubscript{2}CH), 1.43 (s, 9H, t-Bu). ^13C NMR (75 MHz, CDCl\textsubscript{3}, assignments made using DEPT-135) δ: 176.3 (C, C12), 155.8 (C, C13), 136.3 (C, Ar-C), 128.8 (C, Ar-C), 126.3 (CH, Ar-C), 122.4 (CH, Ar-C), 120.0 (CH, Ar-C), 119.3 (CH, Ar-C), 109.8 (C, Ar-C), 109.6 (CH, Ar-C), 80.5 (C, C14), 77.8 (C, N-CH\textsubscript{2}CCH), 73.8 (CH, N-CH\textsubscript{2}CCH), 54.3 (CH, C11), 35.9 (CH\textsubscript{2}, N-CH\textsubscript{2}CCH), 28.4 (3 x CH\textsubscript{3}, C15, C16, C17), 27.7 (CH\textsubscript{2}, C10). MS (ESI) m/z [M-H]\textsuperscript{-} 341.2. Accurate mass calculated for C\textsubscript{19}H\textsubscript{22}N\textsubscript{2}O\textsubscript{4}-H: 341.1507. Found: MS (ESI) 341.1500, error -2.1 ppm.

2.4.15 Boc-D-Trp(N-benzyl)-OH (14)

The method of synthesis was similar to that described for 8; 1 M LiOH was added to 1 g of 7 to give 0.74 g (90.9%) of 14 as a white solid. ^1H NMR (400 MHz, CDCl\textsubscript{3}) δ: 7.61 (d, 1H, J 7.6 Hz, Ar-H), 7.37 (d, 1H, J 4.4 Hz, Ar-H), 7.29-7.22 (m, 3H, Ar-H),
7.16 (t, 1H, J 7.4 Hz, Ar-H), 7.11 (t, 1H, J 6.8 Hz, Ar-H), 7.05 (br-d, 2H, J 7.6 Hz, Ar-H), 6.95 (s, 1H, Ind-2-H), 5.23 (br-s, 2H, N-CH$_2$Ph), 5.05 (br-d, 1H, J 7.6 Hz, NHBoc), 4.70-4.60 (br-m, 1H, CH), 3.37 (dd, 1H, J 14.8, 4.8 Hz, 10-CH$_A$), 3.27 (dd, 1H, J 15.0, 5.8 Hz, 10-CH$_B$), 1.42 (s, 9H, t-Bu). $^{13}$C NMR (75 MHz, CDCl$_3$, assignments made using DEPT-135) $\delta$: 176.8 (C, C$_{12}$), 155.7 (C, C$_{13}$), 137.6 (C, Ar-C on Ind), 136.7 (C, Ar-C), 128.9 (2 x CH, Ar-C on Ind), 128.7 (CH, Ar-C on Ind), 128.6 (C, Ar-C), 127.8 (2 x CH, Ar-C on Ind), 126.8 (CH, Ar-C), 122.1 (CH, Ar-C), 119.6 (CH, Ar-C), 119.1 (CH, Ar-C), 109.9 (CH, Ar-C), 109.3 (C, Ar-C), 80.3 (C, C$_{14}$), 54.4 (CH, C$_{11}$), 50.1 (CH$_2$, N-CH$_2$Ph), 28.4 (3 x CH$_3$, C$_{15}$, C$_{16}$, C$_{17}$), 27.8 (CH$_2$, C$_{10}$). MS (ESI) $m/z$ [M-H]$^{-}$ 393.2. Accurate mass calculated for C$_{23}$H$_{26}$N$_2$O$_4$-H: 393.1820. Found: MS (ESI) 393.1819, error -0.3 ppm.

2.4.16 Fmoc-D-Trp-OH

$^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$: 12.75 (br-s, 1H, -COOH), 10.87 (s, 1H, Ind-1-NH), 7.88 (d, 2H, J 7.2 Hz, Ar-H), 7.72 (d, 1H, J 8.4 Hz, NHFmoc), 7.69-7.64 (m, 2H, Ar-H), 7.57 (d, 1H, J 8.0 Hz, Ar-H), 7.48-7.25 (m, 5H, Ar-H), 7.19 (s, 1H, Ind-2-H), 7.08 (t, 1H, J 7.6 Hz, Ar-H), 6.99 (t, 1H, J 7.4 Hz, Ar-H), 4.33-4.08 (m, 4H, 11-CH, 14-CH$_2$ and 15-CH), 3.21 (dd, 1H, J 14.2, 4.2 Hz, 10-CH$_A$), 3.04 (dd, 1H, J 14.6, 9.8 Hz, 10-CH$_B$).
2.4.17 Fmoc-D-Trp-O-Su (15)

Under inert conditions, DCC (0.97 g, 4.68 mmol) and NHS (0.54 g, 4.68 mmol) were added to a solution of Fmoc-D-Trp (1 g, 2.34 mmol) in THF (30 ml) and stirred at 0 °C to RT overnight. The reaction completion was checked by TLC, eluting with ethyl acetate/hexane (1:1). Dicyclohexylurea (DCU) was formed as a white precipitate and removed by filtration. The filtrate was concentrated under reduced pressure and the residue was purified by column chromatography (ethyl acetate/hexane, 4:1), TLC Rf 0.74. Fractions were collected and concentrated under reduced pressure to give give 1.02 g (83%) of 15 as a white solid. 

$^1$H NMR (400 MHz, DMSO-d$_6$) δ: 10.96 (s, 1H, Ind-1-NH), 8.25 (d, 1H, J 8.0 Hz, NHFmoc), 7.88 (d, 2H, J 7.6 Hz, Ar-H), 7.33-7.24 (m, 3H, Ar-H), 7.10 (t, 1H, J 7.6 Hz, Ar-H), 7.01 (t, 1H, J 7.4 Hz, Ar-H), 4.69-4.64 (m, 1H, 11-CH), 4.23-4.16 (m, 3H, 14-CH$_2$ and 15-CH), 3.38 (dd, 1H, J 14.8, 4.4 Hz, 10-CH$_A$), 3.21 (dd, 1H, J 14.4, 10.0 Hz, 10-CH$_B$), 2.84 (s, 4H, 2 x CH$_2$ of Su). 

$^{13}$C NMR (75 MHz, DMSO-d$_6$, assignments made using DEPT-135) δ: 170.0 (C, C12), 168.4 (2 x C, Su), 155.8 (C, C13), 143.7 (C, Ar-C), 143.6 (C, Ar-C), 140.7 (2 x C, Ar-C), 136.2 (C, Ar-C), 127.6 (2 x CH, Ar-C), 127.1 (2 x CH, Ar-C), 126.8 (C, Ar-C), 125.2 (2 x CH, Ar-C), 124.2 (CH, Ar-C), 121.1 (CH, Ar-C), 120.1 (2 x CH, Ar-C), 118.6 (CH, Ar-C), 117.8 (CH, Ar-C), 111.6 (CH, Ar-C), 108.8 (C, Ar-C), 66.0 (CH$_2$, C14), 53.3 (CH, C11), 46.5 (CH, C15), 27.0 (CH$_2$, C10), 25.5 (2 x CH$_2$, Su). MS (ESI) m/z [M+Na]$^+$ 546.2. Accurate mass calculated for C$_{36}$H$_{22}$N$_3$O$_6$Na: 546.1636. Found: MS (ESI) 546.1634, error -0.4 ppm.
Chapter 2

2.4.18 Fmoc-D-Trp(N-tert-prenyl)-O-Su (16)

![Chemical Structure](image)

Cu(OAc)$_2$ (0.56 g, 3.06 mmol) and AgTFA (0.68 g, 3.06 mmol) were added to a solution of 15 (0.80 g, 1.53 mmol) in dry acetonitrile (MeCN) (20 ml) at RT. 2-Methyl-2-butene (4.86 ml, 45.9 mmol) was added, followed by Pd(OAc)$_2$ (10 mol%, 34.35 mg) at RT. The mixture was heated to 35 ºC, followed by 3 sequential additions of Pd(OAc)$_2$ (10 mol% after each hour x 3). The mixture was allowed to stir for 24 hours at 35 ºC. The reaction completion was checked by TLC, eluting with ethyl acetate/hexane (2:3). The sample was concentrated under reduced pressure and the residue was purified by column chromatography (ethyl acetate/hexane, 2:3), TLC $R_f$ 0.30. Fractions were collected and concentrated under reduced pressure to give 0.3 g (33%) of 16 as a yellow solid. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$: 8.27 (d, 1H, $J$ 8.4 Hz, NHFmoc), 7.88 (d, 2H, $J$ 7.6 Hz, Ar-H), 7.65 (d, 2H, $J$ 7.6 Hz, Ar-H), 7.59 (d, 1H, $J$ 7.6 Hz, Ar-H), 7.45-7.35 (m, 4H, Ar-H), 7.31-7.23 (m, 2H, Ar-H), 7.08-7.00 (m, 2H, Ar-H), 6.07 (dd, 1H, $J$ 17.6, 10.8 Hz, N-C(CH$_3$)$_2$CHC$_2$H$_2$), 5.16 (d, 1H, $J$ 10.8 Hz, N-C(CH$_3$)$_2$CHC$_2$H$_2$), 5.10 (d, 1H, $J$ 17.2 Hz, N-C(CH$_3$)$_2$CHC$_2$H$_2$), 4.72-4.67 (m, 1H, 11-CH$_2$ of Su), 1.66 (s, 3H, N-C(CH$_3$)$_2$CHC$_2$H$_2$), 1.65 (s, 3H, N-C(CH$_3$)$_2$CHC$_2$H$_2$). $^{13}$C NMR (75 MHz, DMSO-$d_6$, assignments made using DEPT-135) $\delta$: 170.0 (C, C12), 168.2 (2 x C, Su), 155.8 (C, C13), 143.9 (CH, Ar-C), 143.7 (C, Ar-C), 143.6 (C, Ar-C), 140.7 (2 x C, Ar-C), 135.0 (C, Ar-C), 128.7 (C, Ar-C), 127.6 (2 x CH, Ar-C), 127.1 (2 x CH, Ar-C), 125.1 (2 x CH, Ar-C), 120.6 (CH, Ar-C), 120.1 (3 x CH, Ar-C), 118.6 (CH, Ar-C), 118.2 (CH$_3$, N-C(CH$_3$)$_2$CHC$_2$H$_2$), 113.6 (CH, Ar-C), 113.3 (CH$_2$, N-C(CH$_3$)$_2$CHC$_2$H$_2$), 107.5 (C, Ar-C), 66.0 (CH$_2$, C14), 58.6 (C, N-C(CH$_3$)$_2$CHC$_2$H$_2$),...
53.2 (CH, C11), 46.5 (CH, C15), 27.4 (CH3, N-C(CH3)2CHCH2), 27.3 (CH3, N-C(CH3)2CHCH2), 27.0 (CH2, C10), 25.5 (2 x CH2, Su). MS (ESI) m/z [M+Na]+ 614.2. Accurate mass calculated for C35H33N3O6Na: 614.2262. Found: MS (ESI) 614.2256, error -1.0 ppm.

2.4.19 Fmoc-D-Trp(N-tert-prenyl)-OH (17)

Sodium carbonate (Na2CO3) (0.11 g, 1 mmol) was added to a solution of 16 (0.15 g, 0.25 mmol) dissolved in 50% v/v MeCN/H2O (16 ml). The retention time (tR) for 16 analysed with RP-HPLC (ACE 5 C8 – 250 X 4.6mm id) is 28.70 minutes at 280 nm. The mixture was stirred overnight at RT. The hydrolysis was checked with RP-HPLC. The reaction mixture was made acidic by addition of excess 1 M HCl and extracted with ethyl acetate (3 x 50 ml). The organic layers were concentrated under reduced pressure. The crude product was purified by RP-HPLC (ACE 10 C4 – 21.2mm id), at 280 nm, with the following non-linear gradient elution: 0 to 65% solution B (0.1% v/v TFA/MeCN) from 0 to 5 minutes, 65 to 95% solution B from 5 to 45 minutes, and 95 to 100% solution B from 45 to 50 minutes, along with solution A (0.1% v/v TFA/H2O), collected, and the main fraction lyophilised to give 65 mg (54%) of 17 as a pale yellow solid. RP-HPLC (ACE 5 C8 – 250 X 4.6mm id) tR 27.15 minutes, at 280 nm. 1H NMR (400 MHz, DMSO-d6) δ: 7.88 (d, 2H, J 7.6 Hz, Ar-H), 7.74 (d, 1H, J 8.0 Hz, NHFmoc), 7.66 (t, 2H, J 6.6 Hz, Ar-H), 7.57 (d, 1H, J 7.6 Hz, Ar-H), 7.43-7.37 (m, 3H, Ar-H), 7.35 (s, 1H, Ind-2-H), 7.29 (t, 1H, J 7.4 Hz, Ar-H), 7.24 (t, 1H, J 7.4 Hz, Ar-H), 7.06-6.98 (m, 2H, Ar-H), 6.07 (dd, 1H, J 17.6, 10.8 Hz, N-C(CH3)2CHCH2), 5.16 (d, 1H, J 10.8 Hz, N-C(CH3)2CHCH2), 5.10 (d, 1H, J 17.2 Hz, N-C(CH3)2CHCH2), 4.27-4.14 (m, 4H, 11-CH, 14-CH2 and 15-CH), 3.30 (t, 2H, J 7.6 Hz, 18-CH2), 3.23 (t, 2H, J 7.6 Hz, 18-CH2), 3.13 (t, 2H, J 7.6 Hz, 18-CH2), 3.03 (t, 2H, J 7.6 Hz, 18-CH2), 2.99 (t, 2H, J 7.6 Hz, 18-CH2), 2.93 (t, 2H, J 7.6 Hz, 18-CH2), 2.81 (t, 2H, J 7.6 Hz, 18-CH2), 2.70 (t, 2H, J 7.6 Hz, 18-CH2), 2.59 (t, 2H, J 7.6 Hz, 18-CH2), 2.49 (t, 2H, J 7.6 Hz, 18-CH2), 2.38 (t, 2H, J 7.6 Hz, 18-CH2), 2.27 (t, 2H, J 7.6 Hz, 18-CH2), 2.16 (t, 2H, J 7.6 Hz, 18-CH2), 2.05 (t, 2H, J 7.6 Hz, 18-CH2), 1.94 (t, 2H, J 7.6 Hz, 18-CH2), 1.83 (t, 2H, J 7.6 Hz, 18-CH2), 1.72 (t, 2H, J 7.6 Hz, 18-CH2), 1.61 (t, 2H, J 7.6 Hz, 18-CH2), 1.50 (t, 2H, J 7.6 Hz, 18-CH2), 1.39 (t, 2H, J 7.6 Hz, 18-CH2), 1.28 (t, 2H, J 7.6 Hz, 18-CH2), 1.17 (t, 2H, J 7.6 Hz, 18-CH2), 1.06 (t, 2H, J 7.6 Hz, 18-CH2), 0.95 (t, 2H, J 7.6 Hz, 18-CH2), 0.84 (t, 2H, J 7.6 Hz, 18-CH2), 0.73 (t, 2H, J 7.6 Hz, 18-CH2), 0.62 (t, 2H, J 7.6 Hz, 18-CH2), 0.51 (t, 2H, J 7.6 Hz, 18-CH2), 0.40 (t, 2H, J 7.6 Hz, 18-CH2), 0.29 (t, 2H, J 7.6 Hz, 18-CH2), 0.18 (t, 2H, J 7.6 Hz, 18-CH2), 0.08 (t, 2H, J 7.6 Hz, 18-CH2), 0.0 (t, 2H, J 7.6 Hz, 18-CH2).
3.20 (dd, 1H, J 14.6, 4.2 Hz, 10-CH₃), 3.01 (dd, 1H, J 14.4, 10.0 Hz, 10-CH₃), 1.65 (s, 3H, N-C(CH₃)₂CHCH₂), 1.64 (s, 3H, N-C(CH₃)₂CHCH₂). ¹³C NMR (75 MHz, DMSO-d₆, assignments made using DEPT-135) δ: 173.7 (C, C₁₂), 156.0 (C, C₁₃), 144.0 (2 x CH, Ar-C), 143.8 (2 x C, Ar-C), 140.7 (2 x C, Ar-C), 135.0 (C, Ar-C), 129.0 (C, Ar-C), 127.6 (2 x CH, Ar-C), 127.0 (2 x CH, Ar-C), 125.2 (CH, Ar-C), 124.5 (CH, Ar-C), 120.4 (CH, Ar-C), 120.1 (2 x CH, Ar-C), 118.5 (CH, Ar-C), 118.4 (CH₃, N-C(CH₃)₂CHCH₂), 113.4 (CH, Ar-C), 113.3 (CH₂, N-C(CH₃)₂CHCH₂), 109.1 (C, Ar-C), 65.7 (CH₂, C₁₄), 58.5 (C, N-C(CH₃)₂CHCH₂), 54.7 (CH, C₁₁), 46.6 (CH, C₁₅), 27.4 (2 x CH₃, N-C(CH₃)₂CHCH₂), 26.8 (CH₂, C₁₀). MS (ESI) m/z [M+H]⁺ 495.4. Accurate mass calculated for C₃₁H₃₀N₂O₄H: 495.2278. Found: MS (ESI) 495.2268, error -2.0 ppm.

2.4.20 D-Trp(N-butyl)-OH (18)

Boc-D-Trp(N-butyl)-OH (11) (66 mg, 0.18 mmol); RP-HPLC (ACE 5 C8 – 250 X 4.6mm id) tᵣ 25.15 minutes at 280 nm, was added to a solution 50% v/v TFA/DCM. The mixture was stirred for 1 hour at RT and then concentrated under reduced pressure. The removal of Boc group was checked with RP-HPLC. The crude product was purified by RP-HPLC (ACE 10 C4 – 250 X 21.2mm id), at 280 nm, with the following non-linear gradient elution: 0 to 25% solution B from 0 to 5 minutes, 25 to 60% solution B from 5 to 45 minutes, and 60 to 100% solution B from 45 to 50 minutes, along with solution A, collected, and the main fraction lyophilised to give 29.1 mg (61.1%) of 18 as a white solid. RP-HPLC (ACE 5 C8 – 250 X 4.6mm id) tᵣ 18.29 minutes, at 280 nm. ¹H NMR (400 MHz, CDCl₃) δ: 7.53 (br-d, 1H, J 6.4 Hz, Ar-H), 7.11 (br-d, 1H, J 6.8 Hz, Ar-H), 7.03 (s, 1H, Ind-2-H), 7.00-6.93 (m, 2H, Ar-H), 4.01-3.87 (m, 3H, 1H of CH and 2H of N-CH₂CH₂CH₂CH₃), 3.27 (dd~br-d, 1H,
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$J$ 13.6 Hz, 10-CH$_A$), 3.09 (dd, 1H, $J$ 14.2, 6.6 Hz, 10-CH$_B$), 1.72 (pentet, 2H, $J$ 7.5 Hz, N-CH$_2$CH$_2$CH$_2$CH$_3$), 1.28 (sextet, 2H, $J$ 7.0 Hz, N-CH$_2$CH$_2$CH$_2$CH$_3$), 0.90 (t, 3H, $J$ 7.2 Hz, N-CH$_2$CH$_2$CH$_2$CH$_3$). $^{13}$C NMR (75 MHz, CDCl$_3$, assignments made using DEPT-135) $\delta$: 173.0 (C, C12), 136.6 (C, Ar-C), 128.6 (C, Ar-C), 127.5 (CH, Ar-C), 122.0 (CH, Ar-C), 119.4 (CH, Ar-C), 118.9 (CH, Ar-C), 109.8 (CH, Ar-C), 105.7 (C, Ar-C), 54.1 (CH, C11), 46.1 (CH$_2$, N-CH$_2$CH$_2$CH$_2$CH$_3$), 32.3 (CH$_2$, N-CH$_2$CH$_2$CH$_2$CH$_3$), 26.2 (CH$_2$, C10), 20.3 (CH$_2$, N-CH$_2$CH$_2$CH$_2$CH$_3$), 13.7 (CH$_3$, N-CH$_2$CH$_2$CH$_2$CH$_3$). MS (ESI) $m/z$ [M-H]$^-$ 259.2. Accurate mass calculated for C$_{13}$H$_{20}$N$_2$O$_2$-H: 259.1452. Found: MS (ESI) 259.1450, error -0.8 ppm.
Chapter 3
Synthesis of Peptides
3. Synthesis of Peptides

3.1 Introduction

The primary amino acid sequence of interest for the novel peptides comprises DMePhe-DTrp-Phe-DTrp(N-R)-Leu-NH$_2$, where R is H or different substituents on the N$_\text{ind}$ of the D-Trp. The substituents were methyl, ethyl, propyl, butyl, pentyl, propargyl, benzyl or tert-prenyl. The C-terminal of the peptide is amidated. SPG (Arg-DTrp-MePhe-DTrp-Leu-Met-NH$_2$) was also synthesised for comparison purposes. Depending on the results obtained from cell viability assays for the above analogues (Chapter 4), optimisation of the most potent peptide was made by modifying the peptide sequence by chain shortening or elongation, and by changing the position of the N$_\text{ind}$-modified amino acid.

Both SPPS and LPPS procedures were used for the synthesis of the peptides, with LPPS being the more common method used. Some of those made with SPPS were repeated with LPPS. This chapter presents the detailed synthesis and characterisation of the peptide analogues.

3.2 Materials and Instrumentation

3.2.1 Materials

The Fmoc and Boc amine-protected amino acids, free amino acids, Rink amide MBHA resin (0.64 mmole/g), 4-methylbenzhydrylamine (MBHA) resin (0.74 mmole/g), and coupling reagents were obtained from Novabiochem, Alfa Aesar, Sigma-Aldrich, Fluorochem, and AGTC Bioproducts. Solvents and other chemicals were obtained from Sigma-Aldrich and Fisher Scientific. The synthesised N$_\text{ind}$-substituted amine protected D-Trp derivatives were used in the peptide synthesis.

3.2.2 Instrumentation

Matrix Assisted Laser Desorption/Ionisation Mass Spectrometry (MALDIMS) (Shimadzu Biotech Axima Confidence) was performed at the School of Chemistry, University of Manchester. SPPS was done manually using a Stuart Scientific wrist action shaker. The Kaiser$^{157}$ test was performed using a Multi-Blok Heater heating
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block. Centrifugations were completed on Hettich Zentrifugen Universal 320 (radius = 15.1 cm) or Eppendorf® MiniSpin (radius = 6.03 cm). RP-HPLC was performed using PerkinElmer Series 200 instruments and analytical chromatograms were generated using TotalChrom 6.3.1 software. The evaporation of solvents, lyophilisation, ESIMS, APCIMS and $^1$H NMR spectroscopy were performed using instruments stated in Section 2.2.2.

3.3 Methods

3.3.1 Solid Phase Peptide Synthesis

3.3.1.1 Fmoc chemistry

Peptides were synthesised from the C-terminal to the N-terminal; the first amino acid coupled had its carboxyl group forming an amide linkage with the resin amine.

The procedure involved the use of a sintered glass reaction vessel (short column) that was silanised by soaking it with 15% v/v dichlorodimethylsilane/toluene overnight. The reason for silanisation is to prevent the adherence of the resin or peptidyl-resin (coupled amino acids attached to resin) to the glass surface. This is done as dichlorodimethylsilane is bonded to free hydroxyl groups on the glass surface. After silanisation, the column was washed with DMF and DCM.

Rink amide MBHA resin was then added and soaked with DCM overnight to swell. DCM was then filtered using a stream of N$_2$ gas to generate positive pressure. Then, the resin was pre-washed twice with DMF. Each wash during the whole synthetic procedure lasts for 2 minutes, with the waste solvent filtered using N$_2$ gas. A typical volume of DMF or DCM is 10 ml for 0.25 g of resin. The amine groups of the Rink amide MBHA resin have Fmoc protection group, which needs to be removed before the synthesis starts. This de-protection step was done using 20% v/v piperidine/DMF solution and agitating the column for 30 minutes. This was followed by four DMF washes. The solution was also used for the de-protection of the Fmoc group of coupled amino acids before subsequent addition of Fmoc protected amino acids.
Amino acid coupling was performed by adding 2 molar equivalents of the protected amino acid and 2 molar equivalents of the coupling reagent, \( N,N,N',N'\text{-tetramethyl-O-(1H-benzotriazol-1-yl)} \)uranium hexafluorophosphate (HBTU) or (7-azabenzo[b]triazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyAOP), dissolved in DMF. Then, 6 molar equivalents of \( N,N\text{-diisopropylethylamine} \) (DIPEA) were added. The numbers of equivalents are relative to the resin substitution based on the theoretical amount of the resultant peptide needed. The column was then shaken for 45 minutes. This was then followed by 2 washes with DMF and 2 washes with DCM.

The Kaiser test was done in an adapted form to check, qualitatively via colour change, if the protected amino acid has been efficiently coupled to free amines on the resin or peptidyl-resin. It can also be used to check the removal of the Fmoc group. The test is sensitive to primary amines but not secondary amines. The resin/peptidyl-resin was washed twice with DCM and dried with \( \text{N}_2 \) gas before the test. The test was performed in a test tube in which few beads of the resin/peptidyl-resin were placed. To the test tube, 100 µl of pyridine and 25 µl of Ninhydrin solution (5% w/v Ninhydrin/ethanol) were added, and then the mixture was heated to 95 °C in a heating block for 5 minutes. As a control, the same procedure was carried out but without the resin/peptidyl-resin.

A yellow colour represents a negative result, in which the primary amine groups are still protected. This colour indicates the efficient coupling of protected amino acids or that the de-protection step needs repeating. The control test also gives a yellow colour. A dark blue colour represents a positive result, in which the primary amine groups are exposed. This shows that the de-protection step was efficiently carried out or that the coupling step needs repeating. When needed, coupling reactions were repeated and if Kaiser test remained positive, the resin is washed with DMF twice and acetylation of peptidyl-resin was performed with 50% v/v acetic anhydride/DMF for 45 minutes, and later washed twice with DMF. This step, known as capping,
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terminates any unreacted primary amine sites to minimise deletion by-products. The steps for amino acid coupling in Fmoc chemistry are summarised in Table 4.

Table 4. Steps for amino acids coupling using Fmoc protection chemistry

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Resin soaked with DCM overnight</td>
</tr>
<tr>
<td>2</td>
<td>DMF Wash (2 x 2 minutes)</td>
</tr>
<tr>
<td>3</td>
<td>20% v/v piperidine/DMF (1 x 30 minutes)</td>
</tr>
<tr>
<td>4</td>
<td>DMF Wash (4 x 2 minutes)</td>
</tr>
<tr>
<td>5</td>
<td>DCM Wash (2 x 2 minutes)</td>
</tr>
</tbody>
</table>
| 6    | Kaiser Test  
   a) If positive = Wash with DMF (2 x 2 minutes) then continue to Step 7  
   b) If negative = Repeat from Step 2 |
| 7    | Coupling Reaction (45 minutes)  
   2 equivalents of Fmoc protected amino acid  
   2 equivalents of HBTU or PyAOP  
   6 equivalents of DIPEA |
| 8    | DMF Wash (2 x 2 minutes) |
| 9    | DCM Wash (2 x 2 minutes) |
| 10   | Kaiser Test  
   a) If positive = Wash with DMF (2 x 2 minutes) then repeat from Step 7  
   b) Remained positive twice, wash with DMF (2 x 2 minutes)  
      then acetylation with 50% v/v acetic anhydride/DMF (1 x 45 minutes) followed by DMF Wash (4 x 2 minutes), then go to Step 3 to couple new amino acid  
   c) If negative = Go to Step 2 to couple new amino acid |
3.3.1.2 Boc chemistry

Boc chemistry employs similar steps to Fmoc chemistry, such as the Kaiser test and the coupling reactions of protected amino acids (Boc protected amino acids), however, it differs in the process of de-protecting the amino acids.

Amino acid coupling requires higher molar equivalents of reagents relative to MBHA resin (1 molar equivalent) to enhance the reaction; 3 molar equivalents of the protected amino acid and HBTU/PyAOP, and 9 molar equivalents of DIPEA. The MBHA resin lacks any protecting group, therefore the coupling of the first protected amino acid can be started directly.

The process of amino acid de-protection was done using 50% v/v TFA/DCM solution. A pre-wash for 5 minutes with this solution was done for the peptidyl-resin before the actual 30 minutes de-protection step. This step was followed by four washes with DCM. At the end of this process, the amine was present as the trifluoroacetate salt. It was neutralised back with 2 washes of 5% v/v DIPEA/DCM followed by 2 DCM washes. Then, the Kaiser test was performed to ensure de-protection. The steps for amino acid coupling in Boc chemistry are summarised in Table 5.
Table 5. Steps for amino acids coupling using Boc protection chemistry

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Resin soaked with DCM overnight</td>
</tr>
<tr>
<td>2</td>
<td>DMF Wash (2 x 2 minutes)</td>
</tr>
</tbody>
</table>
| 3    | Coupling Reaction (45 minutes)  
   3 molar equivalents of Boc protected amino acid  
   3 molar equivalents of HBTU or PyAOP  
   9 molar equivalents of DIPEA |
| 4    | DMF Wash (2 x 2 minutes) |
| 5    | DCM Wash (2 x 2 minutes) |
| 6    | Kaiser Test  
   a) If positive = Wash with DMF (2 x 2 minutes) then repeat from **Step 3**  
   b) Remained positive twice, wash with DMF (2 x 2 minutes) then acetylation with 50% v/v acetic anhydride/DMF (1 x 45 minutes) followed by DMF Wash (4 x 2 minutes) and DCM Wash (2 x 2 minutes), then go to **Step 7**  
   c) If negative = Go to **Step 7** |
| 7    | 50% v/v TFA/DCM (1 x 5 minutes) |
| 8    | 50% v/v TFA/DCM (1 x 30 minutes) |
| 9    | DCM Wash (4 x 2 minutes) |
| 10   | 5% v/v DIPEA/DCM (2 x 2 minutes) |
| 11   | DCM Wash (2 x 2 minutes) |
| 12   | Kaiser Test  
   a) If positive = Wash with DMF (2 x 2 minutes) then repeat from **Step 3** to couple new amino acid  
   b) If negative = Repeat from **Step 7** |
3.3.1.3 Peptide cleavage

3.3.1.3.1 Fmoc chemistry

Cleavage of the peptide from the resin in Fmoc chemistry is performed using TFA. It involves the use of several cleavage mixtures (summarised in Table 6) that have nucleophilic scavengers to prevent side reactions from any reactive species generated during acid treatment. Choice of scavengers used depends on the amino acids present and their side chain protection groups. Any side chains protection groups present were cleaved using TFA.

Table 6. Fmoc Resin Cleavage Mixtures. TFA: trifluoroacetic acid; TIS: triisopropylsilane; EDT: ethandithiol; Arg: Arginine; Met: Methionine; Trp: Tryptophan; Cys: Cysteine; Trt: trityl (triphenylmethyl). Cleavage mixtures adapted from Novabiochem® Fmoc resin cleavage protocols.

<table>
<thead>
<tr>
<th>Cleavage Mixture</th>
<th>Components</th>
<th>Specifications To Use</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td>9.5 ml TFA / 0.25 ml H₂O / 0.25 ml TIS</td>
<td>a) Peptide has no Arg, Met, Trp, Cys or Trt protecting group</td>
</tr>
<tr>
<td><strong>B</strong></td>
<td>5 ml TFA / 0.25 ml H₂O / 0.25 ml Thioanisole / 0.25 ml TIS / 0.125 ml EDT</td>
<td>b) Peptide has Arg, Cys or Met</td>
</tr>
<tr>
<td><strong>C</strong></td>
<td>9.5 ml TFA / 0.25 ml H₂O / 0.25 ml EDT / 0.1 ml TIS</td>
<td>c) Peptide has Trp or Trt protecting group AND has no Arg or Met</td>
</tr>
</tbody>
</table>

The terminal Fmoc protection group was removed before adding the cleavage mixture. The cleavage mixture was added to the column followed by agitation for 2 hours. Then, the mixture was collected in a 50 ml centrifuge tube. Cold diethyl ether (30-35 ml) was added to the tube in order to precipitate the peptide and dissolve the scavengers and by-products. The tube was then centrifuged at 1519.4g for 10 minutes and diethyl ether discarded. Several washes with cold diethyl ether were conducted to remove traces of scavengers and by-products. The residue was left to
dry to give the crude peptide product, which was purified and analysed as described in sections 3.3.3 and 3.3.4.

3.3.1.3.2 Boc chemistry
Cleavage of the peptide from the resin was performed using hydrogen fluoride (HF) gas with p-cresol and thiocresol, as nucleophilic scavengers, in a special apparatus made of polytetrafluoroethylene (PTFE). Scavengers were used for the same purpose as stated above. The terminal Boc group was also removed with HF. Due to the harsh conditions of HF and its harmful effects, the cleavage should be done by authorised and trained personnel. The cleavage was completed by Dr. Harmesh Aojula (Division of Pharmacy and Optometry, University of Manchester).

The peptidyl-resin and scavengers were added to a PTFE vessel and HF gas was condensed with liquid N₂ under a controlled vacuum and left to stir for 1-1.5 hours at 4 °C. HF gas was removed with a stream of N₂ gas into a potassium hydroxide trap. To extract the cleaved peptide, cold diethyl ether (30-35 ml) was added to the PTFE vessel to precipitate the peptide and dissolve the scavengers and by-products, and the mixture was then filtered to recover the cleaved peptide and the resin. Further washes with cold diethyl ether were performed to remove residual scavengers and by-products. Finally, 70% v/v acetic acid/H₂O (10-15 ml) was added to dissolve the peptide, and the solution filtered. Similarly, 80% v/v MeCN/H₂O (30-35 ml) was also added and the solution filtered. The filtrate containing the crude peptide was collected and lyophilised to be purified and analysed as described in sections 3.3.3 and 3.3.4.

Some amino acids used in Boc chemistry have side chain protection groups that are stable to HF. These include formyl in tryptophan and the 2,4-dinitrophenyl (Dnp) in histidine. These protection groups have to be removed before peptide cleavage. Formyl is removed with cold 20% v/v piperidine/DMF for 2 hours at RT and Dnp is removed by thiolysis using 40 mmol 2-mercaptoethanol or thiophenol in DMF for 3-4 hours at RT.¹⁵⁸,¹⁵⁹
3.3.2 Liquid Phase Peptide Synthesis

3.3.2.1 General procedure for peptide synthesis in Liquid Phase Peptide Synthesis

The coupling of amino acids in synthesising peptides using the LPPS procedure can be divided into 2 parts. The first part is the formation of NHS amino acid ester (or short chain NHS peptide ester) as described in Chapter 2. The first amino acid to be activated with NHS is the one from the peptide’s N-terminal. Thus, peptide synthesis moves from N-terminal to C-terminal, opposite to that for SPPS. The second part is the coupling of the unprotected-amine of the amino acid to the NHS activated ester under basic conditions using Na$_2$CO$_3$. The peptides were synthesised by a stepwise coupling without purification of the intermediates.

The NHS ester of the first amino acid was formed with the use of molar equivalents of the amino acid, DCC and NHS dissolved in THF (40-50 ml). After the formation of the NHS ester, DCU was removed by filtration and the filtrate containing the activated ester was collected and kept cooled under ice. The next amino acid in the peptide sequence (2 molar equivalents) and Na$_2$CO$_3$ (8 molar equivalents) were dissolved in a minimal amount of deionised H$_2$O (5-10 ml) and added to the filtrate. The mixture was left overnight at RT. The mixture was then acidified with excess 1 M HCl (400-500 ml) and the precipitate formed was extracted with ethyl acetate (4 x 30 ml). The ethyl acetate layers were collected, washed with deionised H$_2$O and concentrated under reduced pressure. The remaining solid was either dissolved in THF, if a further amino acid in the peptide sequence needed to be coupled, or lyophilised to remove any remaining solvent.

3.3.2.2 Removal of Boc group from the $N^{\text{ind}}$-substituted Boc-D-Trp derivatives and the peptides

The amino acids being coupled in LPPS have free amine group (unprotected), except for the first N-terminal amino acid and the $N^{\text{ind}}$-substituted D-Trp where Boc protection is present. Before coupling the $N^{\text{ind}}$-substituted amino acid, the Boc group was removed with 50% v/v TFA/DCM (5-7 ml) for 1 hour and then the sample was
concentrated under reduced pressure. The Boc group at the peptide's N-terminal was removed at the end of peptide synthesis as previously described. The sample was concentrated under reduced pressure to give the crude peptide for purification and analysis.

### 3.3.2.3 Removal of Fmoc group from the N\textsuperscript{ind}-substituted Fmoc-D-Trp derivative and the peptides

All peptides synthesised by LPPS involved the use of Boc amino acids except for two peptides, in which Fmoc protected amino acids were used. The N-terminal amino acid and the N\textsuperscript{ind}-substituted D-Trp were Fmoc protected. The Fmoc group was removed with 50% v/v diethylamine (DEA)/MeCN\textsuperscript{160} (5-7 ml) for 1 hour and workup procedures were performed as described above to get the crude peptide for purification and analysis.

### 3.3.3 Purification of peptides

Purification of the peptides was performed using RP-HPLC. This involves the use of reversed phase silica columns containing hydrocarbon chains of different lengths. The mechanism of the separation is based on the hydrophobicity/hydrophilicity of the compounds to be purified. An analytical RP-HPLC run was first made to check the purity of the crude peptide. The detector was set at a wavelength of 220 nm. The stationary phase used was a reversed-phase C8 column (ACE 5 C8 – 250 x 4.6 mm id). A few micrograms of the peptide were added to a vial and dissolved in 70% acetic acid in 0.1% v/v TFA/H\textsubscript{2}O (1-1.5 ml). The injection volume used was 50 µl. The mobile phases used for RP-HPLC were 0.1% v/v TFA/H\textsubscript{2}O as solution A and 0.1% v/v TFA/MeCN as solution B. A linear gradient elution was made starting with 100% of Solution A and ending after 30 minutes with 100% of solution B, at a flow rate of 1 ml/min.

Preparative RP-HPLC was performed to purify the crude peptides. This was done using a C4 column (ACE 10 C4 – 250 x 21.2 mm id) with the detector set at 220 nm. The mobile phases were the same as for the analytical RP-HPLC runs. The crude peptides were purified and analysed.
sample to be purified (15-20 mg) was prepared by dissolving in acetic acid and 0.1% v/v TFA/H$_2$O. It was then centrifuged to remove any solid impurities and the supernatant loaded onto the column (maximum 5 ml). The non-linear gradient elution method used was dependent upon the $t_R$ of the peak to be isolated and was developed from the analytical HPLC chromatogram obtained of the crude peptide. The flow rate was 8 ml/min.

For the purified sample, an analytical run was performed as a quality control test to ensure the purity of the peptide and to assign its $t_R$. This was done using the same C8 column and the linear gradient elution program employing the same mobile phases as mentioned above.

### 3.3.4 Characterisation of peptides

The peptides were characterised by MALDIMS using $\alpha$-cyano-4-hydroxycinnamic acid (ACH) matrix, APCIMS, or ESIMS. Some peptides were further characterised by $^1$H NMR spectroscopy.

### 3.4 Results and Discussion

#### 3.4.1 Design and optimisation of peptides

Table 7 shows the primary amino acid sequences for the peptides synthesised using SPPS and LPPS in this project.
Table 7. Primary amino acid sequences of the peptides synthesised using SPPS and LPPS

<table>
<thead>
<tr>
<th>Peptide Number</th>
<th>Peptide Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>SPG / SPPS</td>
</tr>
<tr>
<td>20</td>
<td>Methyl^{4th}-NH₂ / SPPS</td>
</tr>
<tr>
<td>21</td>
<td>Ethyl^{4th}-NH₂ / SPPS</td>
</tr>
<tr>
<td>22</td>
<td>Propyl^{4th}-NH₂ / SPPS</td>
</tr>
<tr>
<td>23</td>
<td>Arg-Butyl^{3rd,5th}-NH₂ / SPPS</td>
</tr>
<tr>
<td>24</td>
<td>Butyl^{4th}-SPG / SPPS</td>
</tr>
<tr>
<td>25</td>
<td>5-mer-NH₂ / LPPS</td>
</tr>
<tr>
<td>26</td>
<td>Methyl^{4th}-NH₂ / LPPS</td>
</tr>
<tr>
<td>27</td>
<td>Ethyl^{4th}-NH₂ / LPPS</td>
</tr>
<tr>
<td>28</td>
<td>Propyl^{4th}-NH₂ / LPPS</td>
</tr>
<tr>
<td>29</td>
<td>Butyl^{4th}-NH₂ / LPPS</td>
</tr>
<tr>
<td>30</td>
<td>Penty^{4th}-NH₂ / LPPS</td>
</tr>
<tr>
<td>31</td>
<td>Propargyl^{4th}-NH₂ / LPPS</td>
</tr>
</tbody>
</table>
3.4.1.1 Synthesis of Substance P antagonist G

There are numerous neuropeptide antagonists that have been synthesised and evaluated in vitro and in vivo against SCLC. Among these peptides is SPG (19, Arg-DTrp-MePhe-DTrp-Leu-Met-NH₂) that entered Phase I clinical trials. This was therefore synthesised again to serve as a reference to compare its cytotoxic activity on SCLC cell lines to the novel peptides. The synthesis of 19 was performed by SPPS using Fmoc chemistry. Fmoc chemistry was chosen as peptides are cleaved from the resin in a simpler way using TFA rather than the harsh conditions of HF in Boc chemistry.

3.4.1.2 Synthesis of the first novel peptide series

The lead peptide (25) comprises DMePhe-DTrp-Phe-DTrp-Leu-NH₂. It is a novel peptide with much homology to NY3460 (DMePhe-DTrp-Phe-DTrp-Leu(CH₂NH)Leu-NH₂) (Chapter 1). Also, 25 was similar to 19 in terms of the presence of N-methylated amino acid (MePhe), D-Trp and Leu residues, and the amide at the C-terminal. Compared to NY3460 and 19, 25 is shorter by a single amino acid. Apart from 19, 25 served as a control peptide to compare its cytotoxicity with the other peptides, of the same sequence, that have different Nⁿ-substituted D-
Trp residues. These modified residues were located near the C-terminal (4\textsuperscript{th} residue), while the D-Trp which is located near the N-terminal remained unmodified (2\textsuperscript{nd} residue). This location was chosen as it could enhance the peptides’ stability and improve cytotoxicity, based on the results with previous SP analogues (SPG (19) and SPD - discussed in Chapter 1). Peptides generated in this series had D-Trp residue substituted with methyl (26), ethyl (27), propyl (28), butyl (29), pentyl (30), propargyl (31), benzyl (32), and tert-prenyl (33). Along with 25, these peptides were synthesised using the LPPS procedure (Table 7).

LPPS was adopted as there was insufficient peptide precipitation when making the peptides bearing methyl (20), ethyl (21) and propyl (22) N\textsuperscript{ind}-modified D-Trp by SPPS. Boc chemistry was chosen as the synthesised N\textsuperscript{ind}-modified D-Trp residues were Boc protected. The reason for poor precipitation was the partial solubility of the N\textsuperscript{ind}-modified D-Trp peptides in diethyl ether, used normally to precipitate the peptides after their cleavage from the resin. Due to the hydrophobicity of these peptides, diethyl ether was able to dissolve a significant portion along with the scavengers and by-products. This can be observed by the relatively very low amounts obtained from the peptides after precipitation, in contrast to 19. The presence of arginine (Arg), which is a charged amino acid, in 19, facilitated its precipitation in diethyl ether.

3.4.1.3 Synthesis of the second novel peptide series
This series consisted of peptides (23, 24 and 34-37) (Table 7) that have N\textsuperscript{ind}-butyl D-Trp residue(s) within their sequence. The butyl group substituent was chosen since 29, which has the butyl group on D-Trp of the 4\textsuperscript{th} position, showed the highest potency among other 4\textsuperscript{th} modified peptides (26-33) and the unmodified peptide (25) against SCLC cell lines tested (Chapter 4). The synthesis of 34-37 was performed using the LPPS procedure, while the synthesis of 23 and 24 was performed using SPPS with Boc chemistry.
Peptide 34 has the modified amino acid’s location changed from 4th residue to 2nd residue, while 35 has both of its D-Trp residues modified. Peptides 34 and 35 were synthesised to observe any change in the cytotoxic activity of the peptides by changing the location of the Nind-modification or when modifying both D-Trp residues. To know whether the terminal amide group or Leu-amide on the C-terminal is important for the peptide activity, 36 and 37 were synthesised, respectively, based on 29. Peptide 37 was synthesised to potentially enhance the solubility of the peptide as Leu, being a hydrophobic amino acid, was removed. The charged amino acid (Arg) was coupled to the N-terminal of 35 to form 23, as another way to enhance the solubility of the peptide.

As peptides bearing the butyl substituent on Nind of their D-Trp residues showed the most potent cytotoxicity (29 and 35 - Chapter 4) against SCLC cell lines, it seems logical to include this modification into 19 that has two D-Trp residues on the 2nd and 4th positions. Peptide 19 had been previously tested before in vitro, in vivo and in Phase I clinical trial, so it is conceivable that inserting an Nind-butyl D-Trp residue might enhance its activity. Peptide 24 was synthesised by modifying the D-Trp residue near the C-terminal (4th position) of 19.

3.4.2 Solid Phase Peptide Synthesis

3.4.2.1 Synthesis of Substance P Antagonist G by Fmoc Chemistry

The primary amino acid sequence of 19 comprises Arg-DTrp-MePhe-DTrp-Leu-Met-NH₂ (Figure 19). Scheme 3 represents the general cycles for SPPS and cleavage using Fmoc protection chemistry. Methionine was the first amino acid to be coupled to the resin in the form of Fmoc-Met-OH. Then, the subsequent amino acids were added in the form of Fmoc-Leu-OH, Fmoc-D-Trp-OH, Fmoc-MePhe-OH, Fmoc-D-Trp-OH and Fmoc-Arg(Pmc)-OH.
Figure 19. The structure of SPG (19) synthesised by Fmoc SPPS
Scheme 3. General scheme for Fmoc SPPS and cleavage. R = amino acid side chain
The Fmoc group of the resin (Figure 20) and of the amino acids was removed by 20% v/v piperidine/DMF. Using the cleavage of Fmoc group of protected amino acid attached to peptide/resin as an example, the general mechanism for Fmoc removal is presented in Scheme 4.161, 162 The piperidine base in the reaction de-protonated the Fmoc group, which eventually led to the formation of carbon dioxide (CO₂), free amine and dibenzofulvene as by-product. In addition, piperidine acted as a nucleophilic scavenger and bound with dibenzofulvene to prevent its side reactions with amino acids.

Figure 20. Rink amide MBHA resin. Nle = Norleucine
During the synthesis, the adapted form of the Kaiser test was performed as described in the methods. Any free primary amines would yield a dark blue chromophore due to their reaction with ninhydrin (Scheme 5). A Schiff’s base (imine) is formed due to the reaction of the amine with the anhydrous keto form of ninhydrin (1,2,3-indantrione), which is in constant equilibrium with its hydrated form. The transformation of the imine would occur due to C–H bond shift (tautomerisation) to give the ketimine, followed by its hydrolysis. The 2-amino intermediate reacts with another molecule of 1,2,3-indantrione to yield the dark blue fully conjugated chromophore, which is called Ruhemann’s purple.

**Scheme 4.** Mechanism for the removal of Fmoc group from protected amino acid attached to peptide/resin. R = amino acid side chain.
HBTU is the coupling reagent that activates the carboxyl end of the amino acid needed for coupling. DIPEA is a base that de-protonates the carboxylic acid to start the reaction. The mechanism for amino acid coupling using HBTU is presented in Scheme 6.\textsuperscript{155, 161, 165} The reaction with HBTU gave an activated amino acid in its benzotriazole form, with tetramethylurea as by-product. The activated amino acid has a highly electron deficient carbonyl group that the amine group (nucleophile) of a resin-bound amino acid attacks to form the amide bond.
An efficient coupling, in which Fmoc protected amino acids are coupled to the free primary amines, would result in a yellow colour in the Kaiser test. This shows that there is no free primary amine on the resin/peptidyl-resin. The de-protection of Fmoc-MePhe-OH would result in a secondary amine. So, even if the coupling of Fmoc-D-Trp-OH was insufficient and free secondary amines were still present, a yellow colour would still be observed in the Kaiser test. This is because the Kaiser test is sensitive to free primary amines (a dark blue colour would be observed) rather than free secondary amine. Hence, the de-protection of Fmoc-MePhe was performed twice to ensure efficient de-protection and Fmoc-D-Trp-OH was coupled to MePhe twice to ensure efficient coupling as well. Apart from Fmoc-D-Trp-OH, the rest of the amino acids were coupled once due to the yellow colour observed in the Kaiser test after each coupling.

The RP-HPLC chromatogram (Figure 21) for the crude sample of peptide 19 synthesised using method A above, showed 2 major peaks at $t_R$ 18.81 ($p_1$) and 20.14 ($p_2$) minutes, together with other impurities.
Figure 21. RP-HPLC chromatogram for the crude product isolated from the synthesis of 19 using method A in SPPS. Peaks p1 and p2 have $t_R$ 18.81 and 20.14 minutes, respectively, at 220 nm, C8 column, acetic acid/H$_2$O solvent. Column was eluted with a 30 minutes linear gradient of 0.1% v/v TFA/H$_2$O and 0.1% v/v TFA/MeCN.

The crude sample was purified and the two peaks were isolated and analysed by MS. The MS results showed an ion at $m/z$: 609.4 for p1 (Figure 22) and 951.5 for p2 (Figure 23).
Figure 22. MALDIMS for p1 showing [M+H]^+: 609.4, [M+Na]^+: 631.4 and [M+K]^+: 647.3.

Figure 23. MALDIMS for p2 showing [M+H]^+: 951.5 and [M+Na]^+: 973.5.

The calculated [M+H]^+ value for 19 is 951.5, hence, p2 was the peak corresponding to 19. The MS result for p1 suggested that it is a deletion by-product, which could have been risen due to the insufficient coupling of Fmoc-D-Trp-OH to MePhe. The calculated [M+H]^+ value for the tetrapeptide is 609.3, hence, p1 corresponds to the tetrapeptide (MePhe-DTrp-Leu-Met-NH₂) as a deletion by-product.
To solve this problem, a stronger coupling reagent was needed to ensure efficient coupling of Fmoc-D-Trp-OH to MePhe. HBTU (a guanidinium salt derived from 1-hydroxybenzotriazole) was among the first safe coupling reagents introduced in SPPS. There are several coupling reagents that are now used in SPPS, including PyAOP, a phosphonium salt derived from 7-aza-hydroxybenzotriazole. PyAOP had higher reactivity in carboxyl activation and amino acid coupling than the other benzo triazole analogues. So, Fmoc-MePhe was de-protected twice and Fmoc-D-Trp-OH was coupled to MePhe once using PyAOP. The rest of the couplings were performed once with HBTU as a yellow colour was observed in the Kaiser test.

The RP-HPLC chromatogram (Figure 24) of the crude sample obtained from the synthesis of 19 using PyAOP (method B) showed a single peak at $t_R$ 21.01 minutes. This method resulted in efficient peptide synthesis without the presence of deletion by-products. The slight difference in $t_R$ is attributed to the fact that a newer C8 column was used for the analysis.

![Figure 24. RP-HPLC chromatogram for the crude product isolated from the synthesis of 19 using method B in SPPS. The peak has a $t_R$ 21.01 minutes, at 220 nm, C8 column, acetic acid/water solvent. Column was eluted with a 30 minutes linear gradient of 0.1% v/v TFA/H$_2$O and 0.1% v/v TFA/MeCN.](image-url)
The sample was purified further (Figure 25) ($t_R$ 20.94 minutes) and MS analysis was performed. MS spectrum (Figure 26) of the pure product showed an ion at $m/z$: 951.7, which corresponds to 19. Accurate mass analysis (Figure 27) was also conducted showing an ion at $m/z$: 951.5033 that corresponds to 19, which has a calculated [M+H]$^+$ of 951.5022 [C$_{49}$H$_{66}$N$_{12}$O$_6$SH].

Figure 25. RP-HPLC chromatogram for 19. $t_R$ 20.94 minutes, at 220 nm, C8 column, acetic acid/water solvent. Column was eluted with a 30 minutes linear gradient of 0.1% v/v TFA/H$_2$O and 0.1% v/v TFA/MeCN.
Chapter 3

Figure 26. MALDIMS for 19 showing [M+H]+: 951.7 and [M+Na]+: 973.6.

Figure 27. Accurate mass for 19 performed using ESIMS showing the observed [M+H]+: 951.5033 (top) and the calculated [M+H]+: 951.5022 (bottom: monoisotopic model).

For the synthesis of 19, the arginine residue was protected as Fmoc-Arg(Pmc)-OH, in which Pmc (2,2,5,7,8-pentamethyl-chroman-6-sulphonyl) is a side chain protection group for arginine guanidinium group. **Cleavage Mixture B** was used to cleave 19.
from the resin. The cleavage mixture contains TFA, which cleaves both the peptide from the resin and Pmc from the arginine. The mixture includes thioanisole, TIS and EDT that scavenge Pmc and any reactive species to prevent their side reactions with the peptide. The peptide was then extracted and purified as explained in the methods.

3.4.2.2 Synthesis of Methyl\(^{4}\text{-NH}_2\), Ethyl\(^{4}\text{-NH}_2\), Propyl\(^{4}\text{-NH}_2\), Arg-Butyl\(^{3,5}\text{-NH}_2\) and Butyl\(^{4}\text{-SPG}\) peptides by Boc Chemistry

The primary amino acid sequence for Methyl\(^{4}\text{-NH}_2\) (20), Ethyl\(^{4}\text{-NH}_2\) (21), and Propyl\(^{4}\text{-NH}_2\) (22) comprises DMePhe-DTrp-Phe-DTrp(N-R)-Leu-NH\(_2\) (Figure 28 a-c), in which R is methyl, ethyl, or propyl group, respectively. The sequence for Arg-Butyl\(^{3,5}\text{-NH}_2\) (23) comprises Arg-DMePhe-DTrp(N-butyl)-Phe-DTrp(N-butyl)-Leu-NH\(_2\) (Figure 28 d) and Butyl\(^{4}\text{-SPG}\) (24) comprises Arg-DTrp-MePhe-DTrp(N-butyl)-Leu-Met-NH\(_2\) (Figure 29). They were synthesised by Boc protection chemistry using SPPS. The resin used was MBHA Resin (Figure 30). Scheme 7 represents the general cycles for SPPS and cleavage using Boc protection chemistry. For 20-23, Leu was the first amino acid to be coupled to the resin in the form of Boc-Leu-OH. Then, the subsequent amino acids were added in the form of Boc-D-Trp(N-methyl, ethyl, propyl, or butyl)-OH, Boc-Phe-OH, Boc-D-Trp(N-For or butyl)-OH, Boc-D-MePhe-OH and Boc-Arg(Tos)-OH. For 24, Boc-Met-OH was first coupled to the resin and the subsequent amino acids were added in the form of Boc-Leu-OH, Boc-D-Trp(N-butyl)-OH, Boc-MePhe-OH, Boc-D-Trp(N-For)-OH and Boc-Arg(Tos)-OH.
Figure 28. Structures of peptides synthesised by SPPS using Boc chemistry

Figure 29. Structure of Butyl$^{4th}$-SPG (24) synthesised by SPPS using Boc chemistry

Figure 30. MBHA resin
Scheme 7. General scheme for Boc SPPS and cleavage. R = amino acid side chain

The mechanism for the cleavage of Boc groups from protected amino acid attached to peptide/resin is presented in Scheme 8. The reaction involved the protonation of the carboxyl group of the carbamate moiety, which led to: 1) the removal of Boc group in the form of CO₂ gas and a tertiary carbocation that de-protonates to form isobutylene gas, and 2) the formation of the trifluoroacetate salt of the amino acid.
The Kaiser test was also performed to monitor the synthesis of peptides as described for the synthesis of 19. Based on the yellow colour observed from the Kaiser test after each coupling, each amino acid was coupled once. This indicated efficient amino acid coupling and absence of uncoupled free amines. To avoid the problem encountered with the synthesis of 19, Boc-D-MePhe in 23 and Boc-MePhe in 24 were de-protected twice and the next amino acid was coupled once with PyAOP.

The formyl (For) side chain protection of the tryptophan amino acid was removed with cold 20% v/v piperidine/DMF for 2 hours and the peptide cleaved from the resin with HF gas as described in the methods. The tosyl (Tos) protection group for the arginine guanidinium group was removed with HF. Thiocresol and p-cresol were added with HF to scavenge any reactive species, such as Tos and carbocations. The peptides were then extracted and purified as described in the methods.
Using the synthesis of \(\mathbf{21}\) as an example, the RP-HPLC chromatogram (Figure 31) for the crude sample showed a major peak at \(t_R\) 23.24 minutes. The crude sample was purified and the main peak was isolated (Figure 32) (\(t_R\) 23.25 minutes) and analysed by MS. MS spectrum (Figure 33) of the pure product showed an ion at \(m/z\): 839.3 that corresponds to \(\mathbf{21}\), which has a calculated [M+H]\(^+\) of 839.5. Accurate mass analysis (Figure 34) was also conducted showing an ion at \(m/z\): 839.4648 that corresponds to \(\mathbf{21}\), which has a calculated [M+H]\(^+\) of 839.4608 [C\(_{40}\)H\(_{58}\)N\(_8\)O\(_5\)H].

**Figure 31.** RP-HPLC chromatogram for the crude product isolated from the synthesis of \(\mathbf{21}\) using SPPS. The peak has a \(t_R\) 23.24 minutes, at 220 nm, C8 column, acetic acid/water solvent. Column was eluted with a 30 minutes linear gradient of 0.1% v/v TFA/H\(_2\)O and 0.1% v/v TFA/MeCN.
Figure 32. RP-HPLC chromatogram for 21. The peak has a $t_R$ 23.25 minutes, at 220 nm, C8 column, acetic acid/water solvent. Column was eluted with a 30 minutes linear gradient of 0.1% v/v TFA/H$_2$O and 0.1% v/v TFA/MeCN.

Figure 33. MALDIMS for 21 showing [M+H]$^+ 839.3$ and [M+Na]$^+ 861.3$. 

-127-
Figure 34. Accurate mass for 21 performed using APCIMS showing the observed [M+H]$^+$: 839.4648 (bottom) and the calculated [M+H]$^+$: 839.4608 (top: monoisotopic model)

3.4.2.3 Peptides synthesised by Solid Phase Peptide Synthesis: purification and characterisation

3.4.2.3.1 Substance P antagonist G (19)

![Substance P antagonist G (19)](image)

The crude product was purified by HPLC using the following non-linear gradient elution: 0 to 35% solution B (0.1% v/v TFA/MeCN) from 0 to 5 minutes, 35 to 70% solution B from 5 to 45 minutes, and 70 to 100% solution B from 45 to 50 minutes, along with solution A (0.1% v/v TFA/water). Peaks were collected, and the main fraction lyophilised to give 19 as a white solid. RP-HPLC $t_R$ 20.94 minutes. MS (ESI) $m/z$ [M+H]$^+$ 951.7. Accurate mass calculated for C_{49}H_{66}N_{12}O_{6}SH: 951.5022. Found: MS (ESI) 951.5033, error 1.2 ppm.
3.4.2.3.2 Methyl<sup>4th</sup>-NH<sub>2</sub> (20)

The crude product was purified by HPLC using the following non-linear gradient elution: 0 to 55% solution B (0.1% v/v TFA/MeCN) from 0 to 5 minutes, 55 to 90% solution B from 5 to 45 minutes, and 90 to 100% solution B from 45 to 50 minutes, along with solution A (0.1% v/v TFA/water). Peaks were collected, and the main fraction lyophilised to give 20 as a white solid. RP-HPLC \( t_R \) 22.58 minutes. MS (MALDI) \( m/z \) [M+H]<sup>+</sup> 825.6. Accurate mass calculated for C<sub>48</sub>H<sub>56</sub>N<sub>8</sub>O<sub>5</sub>H: 825.4446. Found: MS (APCI) 825.4482, error 4.4 ppm.

3.4.2.3.3 Ethyl<sup>4th</sup>-NH<sub>2</sub> (21)

The crude product was purified by HPLC using the non-linear gradient elution as used for peptide 20, to give 21 as a white solid. RP-HPLC \( t_R \) 23.25 minutes. MS (MALDI) \( m/z \) [M+H]<sup>+</sup> 839.3. Accurate mass calculated for C<sub>49</sub>H<sub>58</sub>N<sub>8</sub>O<sub>5</sub>H: 839.4608. Found: MS (APCI) 839.4648, error 4.7 ppm.
3.4.2.3.4 Propyl\textsuperscript{4th}-NH\textsubscript{2} (22)

The crude product was purified by HPLC using the non-linear gradient elution as used for peptide 20, to give 22 as a white solid. RP-HPLC $t_R$ 24.02 minutes. MS (MALDI) $m/z$ [M+H]$^+$ 853.8. Accurate mass calculated for C\textsubscript{50}H\textsubscript{60}N\textsubscript{8}O\textsubscript{5}H: 853.4759. Found: MS (APCI) 853.4772, error 1.5 ppm.

3.4.2.3.5 Arg-Butyl\textsuperscript{3rd,5th}-NH\textsubscript{2} (23)

The crude product was purified by HPLC using the following non-linear gradient elution: 0 to 45% solution B from 0 to 5 minutes, 45 to 80% solution B from 5 to 45 minutes, and 80 to 100% solution B from 45 to 50 minutes, along with solution A. Peaks were collected, and the main fraction lyophilised to give 23 as a white solid. RP-HPLC $t_R$ 25.57 minutes. MS (ESI) $m/z$ [M+H]$^+$ 1079.7. Accurate mass calculated for C\textsubscript{61}H\textsubscript{82}N\textsubscript{12}O\textsubscript{6}H: 1079.6553. Found: MS (ESI) 1079.6547, error -0.6 ppm.
3.4.2.3.6 Butyl\textsuperscript{4th}-SPG (24)

The crude product was purified by HPLC using the following non-linear gradient elution: 0 to 40% solution B from 0 to 5 minutes, 40 to 80% solution B from 5 to 45 minutes, and 80 to 100% solution B from 45 to 50 minutes, along with solution A. Peaks were collected, and the main fraction lyophilised to give 24 as a white solid. RP-HPLC $t_R$ 23.49 minutes. MS (ESI) $m/z$ [M+H]$^+$ 1007.7. Accurate mass calculated for C\textsubscript{53}H\textsubscript{74}N\textsubscript{12}O\textsubscript{6}SH: 1007.5648. Found: MS (ESI) 1007.5633, error -1.5 ppm.

3.4.3 Liquid Phase Peptide Synthesis
3.4.3.1 Synthesis of peptides

The procedure involved the initial synthesis of the NHS amino acid ester (or short chain NHS peptide ester). The NHS ester was then reacted with free amino acid under basic conditions using Na\textsubscript{2}CO\textsubscript{3} to form the amide bond. Scheme 9 represents the mechanism for amino acid coupling using LPPS.\textsuperscript{154} The peptides (Figure 35) were synthesised by stepwise coupling without purification of any intermediates. Final peptide products were purified after removal of the N-terminal Boc or Fmoc group.
Scheme 9. Mechanism for amino acid coupling using LPPS. R = -CHR₁-NH₂ / short peptide, R’ = -CHR₂-COOH, R₁/R₂ = amino acid side chain

5-mer-NH₂ (25) \( R=H \) \( R₁=H \) \( R₂=\text{Leu-NH₂} \)
Methyl⁴ᵗʰ-NH₂ (26) \( R=\text{Me} \) \( R₁=H \) \( R₂=\text{Leu-NH₂} \)
Ethyl⁴ᵗʰ-NH₂ (27) \( R=\text{Et} \) \( R₁=H \) \( R₂=\text{Leu-NH₂} \)
Propyl⁴ᵗʰ-NH₂ (28) \( R=\text{Pr} \) \( R₁=H \) \( R₂=\text{Leu-NH₂} \)
Butyl⁴ᵗʰ-NH₂ (29) \( R=\text{Bu} \) \( R₁=H \) \( R₂=\text{Leu-NH₂} \)
Pentyl⁴ᵗʰ-NH₂ (30) \( R=\text{Pe} \) \( R₁=H \) \( R₂=\text{Leu-NH₂} \)
Propargyl⁴ᵗʰ-NH₂ (31) \( R=\text{CH₂CCH} \) \( R₁=H \) \( R₂=\text{Leu-NH₂} \)
Benzyl⁴ᵗʰ-NH₂ (32) \( R=\text{CH₂Ph} \) \( R₁=H \) \( R₂=\text{Leu-NH₂} \)
tert-Prenyl⁴ᵗʰ-NH₂ (33) \( R=\text{tert-prenyl} \) \( R₁=H \) \( R₂=\text{Leu-NH₂} \)
Butyl²ⁿᵈ-NH₂ (34) \( R=H \) \( R₁=\text{Bu} \) \( R₂=\text{Leu-NH₂} \)
Butyl²ⁿᵈ,⁴ᵗʰ-NH₂ (35) \( R=\text{Bu} \) \( R₁=\text{Bu} \) \( R₂=\text{Leu-NH₂} \)
Butyl⁴ᵗʰ-OH (36) \( R=\text{Bu} \) \( R₁=H \) \( R₂=\text{OH} \)
²ⁿᵈ Butyl⁴ᵗʰ-OH (37) \( R=\text{Bu} \) \( R₁=H \) \( R₂=\text{OH} \)

Figure 35. Structure of peptides synthesised by LPPS

The peptide syntheses for 26-32 and 34-37 were as described in the methods section, starting with the activation of Boc-D-MePhe-OH. The syntheses involved the use of D-Trp-OH, Phe-OH, N⁰-substituted Boc-D-Trp-OH, and Leu-NH₂. Leu instead of
Leu-NH₂ was used to synthesise peptide 36. The Boc group removal was performed as described in the method section using 50% v/v TFA/DCM. Fmoc-D-MePhe-OH was used for the synthesis of 25 and the Fmoc group removal was performed as described in the method section using 50% v/v DEA/DCM.

Using the synthesis of 25 as an example, the RP-HPLC chromatogram (Figure 36) of the crude sample showed a major peak (p) at $t_R$ 21.60 minutes, together with several and other impurities.

![RP-HPLC chromatogram](image)

**Figure 36.** RP-HPLC chromatogram of the crude product from the synthesis of 25. The major peak (p) has a $t_R$ 21.60 minutes, at 220 nm, C8 column, acetic acid/water solvent. The column was eluted with a 30 minutes linear gradient of 0.1% v/v TFA/H₂O and 0.1% v/v TFA/MeCN.

It is clear that there are numerous impurities in the crude sample of 25 obtained after its synthesis using the LPPS procedure. This is in contrast to the crude samples obtained after the SPPS procedure as seen in the synthesis of 19 and 21. Although this can be overcome by purification after each amino acid coupling, this would be very time consuming. This shows the importance of SPPS as it provides fast and efficient peptide synthesis as seen for the synthesis of peptides 19 and 21 when using the appropriate reagents. However, the hydrophobicity of some peptides renders the use of SPPS ineffective, therefore LPPS was chosen. SPPS was an appropriate
method for the syntheses of peptides 19, 23 and 24, since they have the charged amino acid arginine within their sequence. The presence of the arginine enabled efficient peptide precipitation, in contrast to the synthesis of peptides 20, 21 and 22 that lack the arginine amino acid.

The $t_R$ of the methylated (22.58 minutes) (20), ethylated (23.25 minutes) (21) and propylated (24.02 minutes) (22) peptides synthesised using SPPS (previous section) increased as chain lengthened (analysed on the same column with the same mobile phases and elution program). This was expected because by increasing chain length, the hydrophobicity of the peptide increases causing them to elute later on the C8 column (more hydrophobic interactions with the column). Therefore, it can be proposed, that the major peak from this crude sample belonged to the desired peptide (25). Peptide 25 has no alkyl chains and is relatively less hydrophobic than 20, 21 and 22, therefore 25 would be expected to elute earlier than 20. The crude sample was purified and the major peak was isolated (Figure 37) ($t_R$ 21.60 minutes) and analysed by MS. MS spectrum (Figure 38) of the pure product showed an ion at $m/z$: 811.5 that corresponds to 25, which has a calculated [M+H]$^+$ of 811.4. Accurate mass analysis (Figure 39) was also conducted showing an ion at $m/z$: 811.4289 that corresponds to 25, which has a calculated [M+H]$^+$ of 811.4290 [C$_{47}$H$_{54}$N$_8$O$_5$H].
Figure 37. RP-HPLC chromatogram for 25. $t_R$ 21.60 minutes, at 220 nm, C8 column, acetic acid/water solvent. Column was eluted with a 30 minute linear gradient of 0.1% v/v TFA/H$_2$O and 0.1% v/v TFA/MeCN.

Figure 39. Accurate mass for 25 performed using APCIMS showing the observed [M+H]+: 811.4289 (top) and the calculated [M+H]+: 811.4290 (bottom: monoisotopic model)

For the tert-PrenylNH₂ peptide (33), the synthesis was slightly modified. The synthesis was performed using NHS/DCC coupling as before, but two main fragments were first made and then conjugated. Also, Fmoc protected amino acids were used instead of the Boc protected amino acids. The first fragment was the synthesis of the tripeptide; Fmoc-DMePhe-DTrp-Phe-OH, and the second fragment was the dipeptide; Fmoc-D-Trp(N-tert-prenyl)-Leu-NH₂ (Figure 40). Fmoc-D-Trp(N-tert-prenyl)-O-Su (16), which is already in the NHS ester form, was coupled to Leu-NH₂ to form the dipeptide. The Fmoc group of the dipeptide was removed with 50% v/v DEA/MeCN before coupling it to the NHS ester of the tripeptide. The N-terminal Fmoc group of the peptide was also removed as before to form 33.
Figure 40. Structure of Fmoc-D-Trp(N-tert-prenyl)-Leu-NH₂

3.4.3.2 Peptides synthesised by Liquid Phase Peptide Synthesis: purification and characterisation

3.4.3.2.1 5-mer-NH₂ (25)

The crude product was purified by HPLC using the non-linear gradient elution: 0 to 35% solution B (0.1% v/v TFA/MeCN) from 0 to 5 minutes, 35 to 70% solution B from 5 to 45 minutes, and 70 to 100% solution B from 45 to 50 minutes, along with solution A (0.1% v/v TFA/water). Peaks were collected, and the main fraction lyophilised to give 25 as a white solid. RP-HPLC tₚ 21.60 minutes. ¹H NMR (400 MHz, DMSO-d₆) δ: 10.83 (s, 1H, Ind-1-NH), 10.71 (s, 1H, Ind-1’-NH), 8.65 (br-s, 2H, NH₂-amide), 8.54 (d, 1H, J 8.0 Hz, NH-amide), 8.43 (d, 2H, J 8.0 Hz, NH-amide), 8.33 (d, 1H, J 8.4 Hz, Ar-H), 7.73 (d, 1H, J 8.4 Hz, Ar-H), 7.31-6.91 (m, 18H, Ar-H), 4.77-4.65 (m, 3H, CαH), 4.25-4.21 (m, 1H, CαH), 3.76 (br-s, 1H, CαH), 3.12 (dd, 1H, J 14.2, 5.4 Hz, CβH₂), 2.97-2.79 (m, 3H, CβH₂), 2.69-2.64 (m, 2H, CβH₂), 2.45-2.33 (m, 2H, CβH₂), 1.75 (s, 3H, +NH₂CH₃), 1.42-1.30 (m, 3H, 3”-CH and 4”-CH₂), 0.79 (d, 3H, J 6.0 Hz, 1”-CH₃),
0.72 (d, 3H, J 6.0 Hz, 2”-CH₃). MS (APCI) m/z [M+H]+ 811.4. Accurate mass calculated for C₄₇H₅₄N₈O₅H: 811.4290. Found: MS (APCI) 811.4289, error -0.1 ppm.

3.4.3.2.2 Methyl⁴ᵗʰ-NH₂ (26)

The crude product was purified by HPLC using the non-linear gradient elution as for peptide 25, to give 26 as a white solid. RP-HPLC tᵣ 22.51 minutes. MS (APCI) m/z [M+H]+ 825.3. Accurate mass calculated for C₄₈H₅₆N₈O₅H: 825.4446. Found: MS (APCI) 825.4458, error 1.5 ppm.

3.4.3.2.3 Ethyl⁴ᵗʰ-NH₂ (27)

The crude product was purified by HPLC using the non-linear gradient elution as for peptide 25, to give 27 as a white solid. RP-HPLC tᵣ 23.16 minutes. MS (APCI) m/z [M+H]+ 839.7. Accurate mass calculated for C₄₉H₅₈N₈O₅H: 839.4603. Found: MS (APCI) 839.4602, error -0.1 ppm.
3.4.3.4 Propyl\textsuperscript{4th}-NH\textsubscript{2} (28)

The crude product was purified by HPLC using the non-linear gradient elution as for peptide 25, to give 28 as a white solid. RP-HPLC $t_R$ 23.95 minutes. MS (APCI) $m/z$ [M+H]\(^+\) 853.9. Accurate mass calculated for C\(_{50}\)H\(_{60}\)N\(_8\)O\(_5\)H: 853.4759. Found: MS (APCI) 853.4755, error -0.5 ppm.

3.4.3.5 Butyl\textsuperscript{4th}-NH\textsubscript{2} (29)

The crude product was purified by HPLC using the non-linear gradient elution as used for peptide 25, to give 29 as a white solid. RP-HPLC $t_R$ 24.74 minutes. \(^1\)H NMR (400 MHz, DMSO-d\(_6\)) $\delta$: 10.71 (s, 1H, Ind-1’-NH), 8.62 (d, 2H, $J$ 9.2 Hz, NH-amide), 8.55 (d, 1H, $J$ 8.0 Hz, NH-amide), 8.44 (d, 2H, $J$ 8.8 Hz, NH-amide), 8.34 (d, 1H, $J$ 8.4 Hz, NH-amide), 7.74 (d, 1H, $J$ 8.0 Hz, Ar-H), 7.65 (d, 1H, $J$ 7.6 Hz, Ar-H), 7.38 (d, 1H, $J$ 8.0 Hz, Ar-H), 7.29-7.25 (m, 2H, Ar-H), 7.24 (s, 1H, Ind-2’-H), 7.21-6.90 (m, 14H, Ar-H), 4.78-4.67 (m, 3H, CaH), 4.28-4.22 (m, 1H, CaH), 4.04 (t, 2H, $J$ 7.0, N-CH\(_2\)CH\(_2\)CH\(_2\)CH\(_3\)), 3.77 (br-d, 1H, $J$ 6.4 Hz, CaH), 3.11 (dd, 1H, $J$ 14.2, 5.4 Hz, C\(\beta\)H\(_2\)), 2.96-2.81 (m, 3H, C\(\beta\)H\(_2\)), 2.67-2.64 (m, 2H, C\(\beta\)H\(_2\)), 2.45-2.39 (m, 2H, C\(\beta\)H\(_2\)), 1.75 (t, 3H, $J$ 5.2 Hz, \(\text{\textsuperscript{15}NH}_2\text{CH}_3\)), 1.64 (pentet, 2H, $J$ 7.4 Hz, N-
CH$_2$CH$_2$CH$_3$), 1.41-1.34 (m, 3H, 3"'-CH and 4"'-CH$_2$), 1.19 (sextet, 2H, $J$ 7.4 Hz, N-CH$_2$CH$_2$CH$_2$CH$_3$), 0.80-0.71 (m, 9H, 6H of 1"'-CH$_3$ and 2"'-CH$_3$ and 3H of N-CH$_2$CH$_2$CH$_2$CH$_3$). MS (APCI) $m/z$ [M+H]$^+$ 867.7. Accurate mass calculated for C$_{51}$H$_{62}$N$_8$O$_5$H: 867.4916. Found: MS (APCI) 867.4927, error 1.3 ppm.

3.4.3.2.6 Pentyl$^{4\text{th}}$-NH$_2$ (30)

The crude product was purified by HPLC eluting with a non-linear gradient: 0 to 50% solution B from 0 to 5 minutes, 50 to 85% solution B from 5 to 45 minutes, and 85 to 100% solution B from 45 to 50 minutes, along with solution A. Peaks were collected, and the main fraction lyophilised to give 30 as a white solid. RP-HPLC $t_R$ 25.61 minutes. MS (APCI) $m/z$ [M+H]$^+$ 881.5. Accurate mass calculated for C$_{52}$H$_{64}$N$_8$O$_5$H: 881.5072. Found: MS (APCI) 881.5068, error -0.5 ppm.

3.4.3.2.7 Propargyl$^{4\text{th}}$-NH$_2$ (31)

The crude product was purified by HPLC using the non-linear gradient elution as used for 25, to give 31 as a white solid. RP-HPLC $t_R$ 22.67 minutes. MS (APCI) $m/z$
3.4.3.2.8 Benzyl\textsuperscript{4\texttext{-th}}-NH\textsubscript{2} (32)

The crude product was purified by HPLC using the non-linear gradient elution as used for peptide 25, to give 32 as a white solid. RP-HPLC \( t_R \) 24.38 minutes. MS (APCI) \( m/z [\text{M+H}]^+ \) 901.7. Accurate mass calculated for C\textsubscript{54}H\textsubscript{60}N\textsubscript{8}O\textsubscript{5}H: 901.4759. Found: MS (APCI) 901.4780, error 2.3 ppm.

3.4.3.2.9 Fmoc-D-Trp(N-\text{tert-prenyl})-Leu-NH\textsubscript{2} (dipeptide for the synthesis of 33)

The crude product was purified by HPLC eluting with the following non-linear gradient: 0 to 60% solution B from 0 to 5 minutes, 60 to 80% solution B from 5 to 45 minutes, and 80 to 100% solution B from 45 to 50 minutes, along with solution A, and the main fraction lyophilised to give Fmoc-D-Trp(N-\text{tert-prenyl})-Leu-NH\textsubscript{2} as a white solid (0.1 g, 47.06%). RP-HPLC \( t_R \) 27.20 minutes. \(^1\text{H} \text{NMR} (400 \text{ MHz, DMSO-}d_6) \delta: 8.19 (d, 1H, J 8.4 Hz, NH-amide), 7.88 (d, 2H, J 7.6 Hz, Ar-H), 7.71-7.67 (m, 3H, NH-amide and Ar-H), 7.62 (d, 1H, J 7.6 Hz, Ar-H), 7.43-7.38 (m, 4H,
1H of NH$_2$-amide and 3H of Ar-H), 7.30 (t, 1H, J 7.4 Hz, Ar-H), 7.27-7.23 (m, 2H, Ar-H), 7.04-6.96 (m, 3H, 1H of NH$_2$-amide and 2H of Ar-H), 6.06 (dd, 1H, J 17.4, 10.6 Hz, N-C(CH$_3$)$_2$CHCH$_2$), 5.16 (d, 1H, J 10.8 Hz, N-C(CH$_3$)$_2$CHCH$_2$), 5.10 (d, 1H, J 17.6 Hz, N-C(CH$_3$)$_2$CHCH$_2$), 4.33 (q, 1H, J 7.5 Hz, 16-CH), 4.17 (br-s, 4H, 11-CH$_2$ and 15-CH), 3.09 (dd, 1H, J 14.2, 6.6 Hz, 10-CH$_A$), 2.92 (dd, 1H, J 14.4, 8.4 Hz, 10-CH$_B$), 1.66 (s, 3H, N-C(CH$_3$)$_2$CHCH$_2$), 1.64 (s, 3H, N-C(CH$_3$)$_2$CHCH$_2$), 1.40-1.28 (m, 3H, 17-CH$_2$ and 18-CH), 0.76 (d, 3H, J 6.0 Hz, 19-CH$_3$), 0.67 (d, 3H, J 6.0 Hz, 20-CH$_3$).

$^{13}$C NMR (75 MHz, DMSO-d$_6$, assignments made using DEPT-135) $\delta$ 174.1 (C, C$_{12}$), 171.5 (C, C$_{21}$), 156.0 (C, C$_{13}$), 144.0 (CH, Ar-C), 143.8 (C, Ar-C), 143.7 (C, Ar-C), 140.7 (2 x C, Ar-C), 134.9 (C, Ar-C), 129.2 (C, Ar-C), 127.6 (2 x CH, Ar-C), 127.0 (2 x CH, Ar-C), 125.3 (2 x CH, Ar-C), 124.7 (CH, Ar-C), 120.3 (CH, Ar-C), 120.1 (3 x CH, Ar-C), 118.8 (CH, Ar-C), 118.2 (CH$_3$, N-C(CH$_3$)$_2$CHCH$_2$), 113.2 (CH$_2$, N-C(CH$_3$)$_2$CHCH$_2$), 108.8 (C, Ar-C), 65.8 (CH$_2$, C$_{14}$), 58.5 (C, N-C(CH$_3$)$_2$CHCH$_2$), 55.7 (CH, C$_{11}$), 50.8 (CH, C$_{16}$), 46.6 (CH, C$_{15}$), 40.7 (CH$_2$, C$_{17}$), 27.6 (CH$_2$, C$_{10}$), 27.5 (CH$_3$, N-C(CH$_3$)$_2$CHCH$_2$), 27.4 (CH$_3$, N-C(CH$_3$)$_2$CHCH$_2$), 24.1 (CH, C$_{18}$), 23.0 (CH$_3$, C$_{19}$), 21.3 (CH$_3$, C$_{20}$). MS (ESI) m/z [M+Na]$^+$ 629.3. Accurate mass calculated for C$_{37}$H$_{42}$N$_4$O$_4$Na: 629.3098. Found: MS (ESI) 629.3097, error -0.2.

3.4.3.2.10 tert-Prenyl$^{4\text{th}}$-NH$_2$ (33)

The crude product was purified by HPLC using the non-linear gradient elution as for peptide 24, to give 33 as a yellow solid. RP-HPLC $t_R$ 24.83 minutes. $^1$H NMR (500 MHz, DMSO-d$_6$) $\delta$: 10.70 (s, 1H, Ind-1’-NH), 8.67 (br-s, 1H, NH-amide), 8.61 (d, 1H, J 9.0 Hz, NH-amide), 8.57 (d, 1H, J 8.0 Hz, NH-amide), 8.40 (t, 3H, J 7.5 Hz, ...
NH-amide and NH$_2$-amide), 7.77 (d, 1H, $J$ 6.5 Hz, Ar-H), 7.65 (d, 1H, $J$ 7.5 Hz, Ar-H), 7.45 (s, 1H, Ind-2-H), 7.38 (d, 1H, $J$ 7.0 Hz, Ar-H), 7.31 (s, 1H, Ind-2'-H), 7.26 (d, 1H, $J$ 8.0 Hz, Ar-H), 7.21-7.17 (m, 3H, Ar-H), 7.05-6.96 (m, 11H, Ar-H), 6.00 (dd, 1H, $J$ 14.0, 8.4 Hz, N-C(CH$_3$)$_2$CHCH$_2$), 5.11 (d, 1H, $J$ 8.8 Hz, N-C(CH$_3$)$_2$CHCH$_2$), 5.10 (d, 1H, $J$ 14.0 Hz, N-C(CH$_3$)$_2$CHCH$_2$), 4.77-4.72 (m, 3H, CαH), 4.31-4.26 (m, 1H, CαH), 3.76 (br-d, 1H, $J$ 5.2 Hz, CαH), 3.13 (dd, 1H, $J$ 14.0, 5.0 Hz, CβH$_2$), 2.95-2.80 (m, 3H, CβH$_2$), 2.65-2.63 (m, 2H, CβH$_2$), 2.43-2.36 (m, 2H, CβH$_2$), 1.75 (t, 3H, $J$ 5.0 Hz, +NH$_2$CH$_3$), 1.65 (s, 3H, N-C(CH$_3$)$_2$CHCH$_2$), 1.60 (s, 3H, N-C(CH$_3$)$_2$CHCH$_2$), 1.49-1.37 (m, 3H, 3-CH and 4-CH$_2$), 0.82 (d, 3H, $J$ 6.5 Hz, 1-CH$_3$), 0.77 (d, 3H, $J$ 6.5 Hz, 2-CH$_3$). MS (ESI) $m/z$ [M+H]$^+$ 879.4. Accurate mass calculated for C$_{52}$H$_{62}$N$_8$O$_5$H: 879.4916. Found: MS (ESI) 879.4916, error 0.0 ppm.

3.4.3.2.11 Butyl$^{2}$-NH$_2$ (34)

The crude product was purified by HPLC using the non-linear gradient elution as used for peptide 25, to give 34 as a white solid. RP-HPLC $t_R$ 24.17 minutes. $^1$H NMR (400 MHz, DMSO-d$_6$) δ: 10.82 (s, 1H, Ind-1-NH), 8.66 (d, 2H, $J$ 8.8 Hz, NH$_2$-amide), 8.52 (d, 1H, $J$ 7.6 Hz, NH-amide), 8.40 (d, 2H, $J$ 8.8 Hz, NHamide), 8.30 (d, 1H, $J$ 8.4 Hz, NH-amide), 7.73 (d, 1H, $J$ 7.6 Hz, Ar-H), 7.64 (d, 1H, $J$ 7.6 Hz, Ar-H), 7.34-6.89 (m, 18H, Ar-H), 4.79-4.64 (m, 3H, CαH), 4.27-4.21 (m, 1H, CαH), 4.06-3.90 (m, 2H, N-CH$_2$CH$_2$CH$_2$CH$_3$), 3.77 (br-d, 1H, $J$ 6.8 Hz, CaH), 3.12 (dd, 1H, $J$ 14.4, 5.6 Hz, CβH$_2$), 2.97-2.81 (m, 3H, CβH$_2$), 2.71-2.63 (m, 2H, CβH$_2$), 2.46-2.28 (m, 2H, CβH$_2$), 1.77 (t, 3H, $J$ 5.0 Hz, +NH$_2$CH$_3$), 1.62 (pentet, 2H, $J$ 7.3 Hz, N-CH$_2$CH$_2$CH$_2$CH$_3$), 1.38-1.33 (m, 3H, 3"-CH and 4"-CH$_2$), 1.23 (sextet, 2H, $J$ 7.4 Hz,
N-CH₂CH₂CH₃), 0.86 (t, 3H, J 7.2 Hz, N-CH₂CH₂CH₃), 0.79 (d, 3H, J 6.4 Hz, 1''-CH₃), 0.72 (d, 3H, J 6.0 Hz, 2''-CH₃). MS (ESI) m/z [M+H]+ 867.5. Accurate mass calculated for C₅₁H₆₂N₈O₅H: 867.4916. Found: MS (ESI) 867.4912, error -0.5 ppm.

3.4.3.2.12 Butyl₂nd,₄th-:NH₂ (35)

The crude product was purified by HPLC eluting with the following non-linear gradient: 0 to 45% solution B from 0 to 5 minutes, 45 to 90% solution B from 5 to 45 minutes, and 90 to 100% solution B from 45 to 50 minutes, along with solution A. Peaks were collected, and the main fraction lyophilised to give 35 as a white solid. RP-HPLC tᵣ 27.75 minutes. ¹H NMR (400 MHz, DMSO-d₆) δ: 8.64 (d, 2H, J 9.2 Hz, NH₂-amide), 8.54 (d, 2H, J 8.0 Hz, NH-amide), 8.41 (d, 1H, J 8.8 Hz, NH-amide), 8.33 (d, 1H, J 8.8 Hz, NH-amide), 7.74 (d, 1H, J 7.6 Hz, Ar-H), 7.64 (d, 1H, J 8.0 Hz, Ar-H), 7.37 (d, 1H, J 8.4 Hz, Ar-H), 7.33 (d, 1H, J 8.4 Hz, Ar-H), 7.29-6.95 (m, 14H, Ar-H), 6.90 (s, 2H, Ind-2'-H and Ind-2'-H), 4.78-4.67 (m, 3H, CaH), 4.27-4.22 (m, 1H, CaH), 4.05-3.95 (m, 4H, 1N-CH₂CH₂CH₂CH₃ and 1'N-CH₂CH₂CH₂CH₃), 3.76 (br-s, 1H, CaH), 3.11 (dd, 1H, J 14.2, 5.4 Hz, CβH₂), 2.96-2.79 (m, 3H, CβH₂), 2.70-2.63 (m, 2H, CβH₂), 2.47-2.38 (m, 2H, CβH₂), 1.77 (br-s, 3H, "NH₂CH₃"), 1.67-1.58 (m, 4H, 1N-CH₂CH₂CH₂CH₃ and 1'N-CH₂CH₂CH₂CH₃), 1.39-1.34 (m, 3H, 3''-CH and 4''-CH₂), 1.28-1.16 (m, 4H, 1N-CH₂CH₂CH₂CH₃ and 1'N-CH₂CH₂CH₂CH₃), 0.85 (t, 3H, J 7.2 Hz, 1''-CH₂CH₂CH₂CH₃), 0.79-0.71 (m, 9H, 6H of 1''-CH₃ and 2''-CH₃ and 3H of 1N-CH₂CH₂CH₂CH₃). MS (ESI) m/z [M+H]+ 923.5. Accurate mass calculated for C₅₅H₇₀N₈O₅H: 923.5542. Found: MS (ESI) 923.5537, error -0.5 ppm.
3.4.3.2.13 Butyl\textsuperscript{4th}-OH (36)

The crude product was purified by HPLC eluting with the following non-linear gradient: 0 to 40% solution B from 0 to 5 minutes, 40 to 75% solution B from 5 to 45 minutes, and 75 to 100% solution B from 45 to 50 minutes, along with solution A. Peaks were collected, and the main fraction lyophilised to give 36 as a white solid. RP-HPLC $t_R$ 25.49 minutes. \textsuperscript{1}H NMR (400 MHz, DMSO-d\textsubscript{6}) $\delta$: 12.52 (br-s, 1H, \text{-COOH}), 10.69 (br-s, 1H, Ind-1'-NH), 8.60-8.52 (m, 3H, NH-amide), 8.44 (d, 1H, J 8.8 Hz, NH-amide), 7.77 (d, 1H, J 7.6 Hz, Ar-H), 7.65 (d, 1H, J 8.0 Hz, Ar-H), 7.37 (d, 1H, J 8.0 Hz, Ar-H), 7.26 (d, 1H, J 8.0 Hz, Ar-H), 7.23 (s, 1H, Ind-2'-H), 7.18-6.91 (m, 15H, Ar-H), 4.85-4.69 (m, 3H, C\textalpha{}H), 4.31-4.25 (m, 1H, C\textalpha{}H), 4.03 (t, 2H, J 7.2 Hz, N-CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{3}), 3.75 (br-d-s, 1H, C\textalpha{}H), 3.11 (dd, 1H, J 14.4, 4.8 Hz, C\textbeta{}H), 2.96-2.80 (m, 3H, C\textbeta{}H), 2.67-2.60 (m, 2H, C\textbeta{}H), 2.41-2.32 (m, 2H, C\textbeta{}H), 1.72 (t, 3H, J 5.0 Hz, $^4$NH\textsubscript{2}CH\textsubscript{3}, on addition of D\textsubscript{2}O t is converted to s), 1.62 (pentet, 2H, J 7.4 Hz, N-CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{3}), 1.49-1.47 (m, 3H, 3”-CH and 4”-CH\textsubscript{2}), 1.20 (sextet, 2H, J 7.5 Hz, N-CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{3}), 0.82 (d, 3H, J 6 Hz, N-CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{3}), 0.78-0.73 (m, 6H, 1”-CH\textsubscript{3} and 2”-CH\textsubscript{3}). MS (ESI) m/z [M+H]\textsuperscript{+} 868.4. Accurate mass calculated for C\textsubscript{51}H\textsubscript{61}N\textsubscript{7}O\textsubscript{6}H: 868.4756. Found: MS (ESI) 868.4752, error -0.5 ppm.
3.4.3.2.14 2\textsuperscript{nd} Butyl\textsuperscript{4th}-OH (37)

The crude product was purified by HPLC using the non-linear gradient elution as used for peptide 25, to give 37 as a white solid. RP-HPLC $t_R$ 23.93 minutes. MS (ESI) $m/z$ [M+H]$^+$ 755.4. Accurate mass calculated for C$_{45}$H$_{50}$N$_6$O$_5$H: 755.3915. Found: MS (ESI) 755.3909, error -0.8 ppm.
Chapter 4
Gastrin Releasing Peptide Receptor Expression, Cell Viability Assays and Assessment of Apoptosis
4. Gastrin Releasing Peptide Receptor Expression, Cell Viability Assays and Assessment of Apoptosis

4.1 Introduction

There are several neuropeptide growth factors and receptors involved in the proliferation of SCLC. These neuropeptides include GRP, NMB, CCK, NT, AVP, BK and galanin and they act through autocrine growth loops via interaction with their receptors (Chapter 1). GRPR are known to be overexpressed in several human cancers, such as lung, prostate, breast, gastric, pancreatic cancers and gastrointestinal carcinoid tumours. GRPR was shown to be expressed in 85% of patients with SCLC (17/20 cases) and 85% of patients with NSCLC (11/13 cases). The expression of GRPR had been shown to increase in SCLC cells as they become resistant to first line chemotherapy. The increase in GRPR expression also enhanced the sensitivity of these cells to SPG (19). Hence, SCLC cell lines that are intended to be used for the assays were checked for their GRPR expression.

This chapter has the following experimental plan:

a) Check the expression of GRPR in SCLC cells using western blot technique. Western blot is used to separate protein mixtures (obtained from cell lysates) according to their molecular weight through electrophoresis.

b) Evaluate the cytotoxicity (IC$_{50}$) of the peptides on the SCLC cells (in vitro) via cell viability assays. These assays were performed in vitro on SCLC cell lines using the resazurin dye solution. It is a simple, one-step, fast and sensitive method for determining cell viability. It is inexpensive and avoids the drawbacks of other methods used for cell viability assays. When the dye is applied, viable cells reduce resazurin (oxidised, non-fluorescent, blue colour) into resorufin (reduced, fluorescent, pink colour) (Scheme 10).
Scheme 10. Resazurin reduction by viable cells into resorufin

c) Check whether the peptides exert their cytotoxic effect via inducing apoptosis. This was done using fluorescent techniques. A qualitative assessment of apoptosis was carried out using Acridine Orange (AO) and Ethidium Bromide (EB) dual staining with the use of an inverted fluorescence microscope. A quantitative measurement was performed using Annexin V conjugate with the use of flow cytometry. AO/EB dual staining can detect different fluorescence emissions due to entry of AO, EB or both depending on the integrity of the plasma membrane, as well as highlighting the shape of the cells. Annexin V binds to phosphatidyl serine (PS), which is expressed only on the outer surface of plasma membranes of cells undergoing apoptosis.

4.2 Materials and Instrumentation

4.2.1 Materials

Growth media, RPMI-1640 (with L-glutamine and NaHCO₃), Dulbecco’s PBS (without calcium chloride and magnesium chloride), Trypan Blue dye, Resazurin sodium salt and Tris Buffered Saline (tablet for 500 ml) were obtained from Sigma-Aldrich. Human Caucasian lung small cell carcinoma (H69 (ECACC 91091802)) cell line was obtained from the European Collection of Authenticated Cell Cultures (ECACC) [Public Health England]. Human lung small cell carcinoma (DMS79) cell line was available at the University of Manchester. Heat Inactivated Fetal Bovine Serum (FBS) was obtained from Life Technologies™. The 96 and 12 well cell culture plates and tissue culture flasks (T25, T75, and T225), Corning® 25 cm², 75 cm² and 225 cm² flasks, were obtained from Corning. The primary antibody for GRPR [GRPR (D-1): sc-398549], primary antibody for β-Actin [β-Actin (C4): sc-47778], the positive control lysate [MIA PaCa-2 Cell Lystae: sc-2285], the secondary
antibody [goat anti-mouse IgG-HRP: sc-2031], and RIPA Lysis Buffer System [sc-24948] were obtained from Santa Cruz Biotechnology. The PageRuler\textsuperscript{TM} Prestained Protein Ladder [26616] was obtained from ThermoFisher Scientific. Clarity\textsuperscript{TM} Western ECL Substrate [170-5061] was obtained from Bio-Rad. Immobilon\textsuperscript{®}-P PVDF transfer membrane was obtained from Merck Millipore. NALGENE\textsuperscript{TM} Cryo 1°C Freezing Container and Star Lab 1.8 ml cryogenic vials were used for cells preservation. Acridine Orange was obtained from Alfa Aesar. Ethidium Bromide was obtained from Fluorochem. Annexin V conjugate (Alexa Fluor\textsuperscript{®} 555), SYTOX\textsuperscript{®} Blue Dead Cell Stain and Annexin-binding Buffer for Flow Cytometry were obtained from Invitrogen\textsuperscript{™}. Other materials were obtained from Sigma Aldrich and Fisher Scientific.

4.2.2 Instrumentation

Absorbance readings were recorded on a UV/VIS Spectrophotometer Perkin Elmer Lambda 12 instrument. Fluorescence readings were recorded on a Tecan Safire plate reader. A Stuart\textsuperscript{®} rotator was used during cell lysis. Bio-Rad Mini Protean\textsuperscript{®} II system was used for protein electrophoresis and transfers. Protein sample denaturing was done on AccuBlock\textsuperscript{TM} Digital Dry Bath. Membranes shaking were performed using Stuart\textsuperscript{®} shaker. Bands on membranes were visualised with ChemiDoc\textsuperscript{TM} MP Imaging System (Bio-Rad). The cells were incubated using HERA cell 150 – Thermo Scientific incubator. Gilson\textsuperscript{®} pipettes P2 / P20 / P200 / P1000N were used. A Grant water bath was used. Cell work was performed in Walker class II safety cabinets. For assessment of apoptosis using AO/EB dual staining, images were collected on an Olympus IX83 inverted microscope using a 10x / 0.3 UPlanFL N objective lens and captured using an Ocra ER camera (Hamamatsu) through CellSens software (Olympus). Specific band pass filter sets for FITC and Texas Red were used to prevent bleed through from one channel to the next. Images were then processed and analysed using Fiji ImageJ. The flow cytometer used for Annexin V conjugate analysis was BD Biosciences - LSR Fortessa - Diva 8.0.1 software; Annexin V conjugate was excited with 561 nm laser and fluorescence measured using a 586/15 nm bandpass filter; dead cell stain was excited with 405 nm laser and fluorescence
measured using a 450/50 nm bandpass filters. Centrifugation was performed using instruments stated in Section 3.2.2.

4.3 Methods

4.3.1 Western blot

4.3.1.1 Buffers and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis gel preparations

4.3.1.1.1 Western blot running buffer

30.0 g of Tris base, 144.0 g of glycine, and 10.0 g of sodium dodecyl sulphate (SDS) were mixed in distilled water to a final volume of 1 L to obtain 10X running buffer. 1X running buffer was used for western blot by mixing 100 ml of 10X with 900 ml of distilled water.

4.3.1.1.2 Western blot transfer buffer

33.0 g of Tris base and 112.5 g of glycine were mixed in distilled water to a final volume of 1 L to obtain 10X transfer buffer. 1X transfer buffer was used for western blot by mixing 100 ml of 10X, 200 ml of methanol, and 700 ml of distilled water.

4.3.1.1.3 Sample buffer

3X sample buffer was composed of 4.8 ml of 1 M Tris-HCl pH 6.8, 6 ml of 20% SDS, 6 ml of glycerol, 3.2 ml of 2-mercaptoethanol, and 12.0 mg of bromophenol blue mixed in distilled water to a final volume of 20 ml. The glycerol was needed to ease the sinking of the samples into the gel’s wells. Bromophenol blue was used as a tracking dye for band movement and separation.\(^{168}\)

4.3.1.1.4 Tris Buffered Saline-Tween

Two tablets of Tris Buffered Saline (TBS) and 1 ml of Tween 20 were mixed in distilled water to a final volume of 1 L (0.15 M sodium chloride; 0.050 M Tris-HCl; pH 7.6).
4.3.1.1.5 Blocking buffer

The blocking buffer was added to the membrane to block non-specific binding sites and to dilute the primary and secondary antibodies. It was prepared by dissolving 5% w/v non-fat dried milk powder in TBS-Tween.

4.3.1.1.6 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis gel

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) gels are usually prepared using variable concentrations of acrylamide based on the size of proteins to be resolved. The lower the molecular weight of the protein, the higher the concentration of acrylamide to be used. Higher concentration of acrylamide results in a smaller pore size, suited to resolve low molecular weight proteins.

Three common gels with acrylamide concentrations 7.5%, 10%, and 15% are usually prepared. The gel is composed of two parts: the resolving and the stacking gels. The stacking gel is slightly acidic and is used to form sharp and thin bands whereas the resolving gel is basic, with higher acrylamide content, in which protein separation takes place. The recipe for typical gel preparation for each of the acrylamide concentrations stated is presented in Table 8.

Table 8. SDS-PAGE Gels Composition. EDTA: Ethylenediaminetetraacetic acid; SDS: Sodium dodecyl sulfate; APS: Ammonium persulfate; TEMED: Tetramethylethylenediamine

<table>
<thead>
<tr>
<th>Gels</th>
<th>7.5% Gel</th>
<th>10% Gel</th>
<th>15% Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solutions</td>
<td>Resolving</td>
<td>Stacking</td>
<td>Resolving</td>
</tr>
<tr>
<td>Water (ml)</td>
<td>4.43</td>
<td>2.24</td>
<td>3.65</td>
</tr>
<tr>
<td>Acrylamide (ml)</td>
<td>2.33</td>
<td>0.56</td>
<td>3.11</td>
</tr>
<tr>
<td>1.5 M Tris pH 8.95 (ml)</td>
<td>2.33</td>
<td>2.33</td>
<td>2.33</td>
</tr>
<tr>
<td>1 M Tris pH 6.95 (ml)</td>
<td></td>
<td>0.42</td>
<td>0.42</td>
</tr>
<tr>
<td>0.2 M EDTA (µl)</td>
<td>93.33</td>
<td>33.33</td>
<td>93.33</td>
</tr>
<tr>
<td>10% SDS (µl)</td>
<td>93.33</td>
<td>33.33</td>
<td>93.33</td>
</tr>
<tr>
<td>10% APS (µl)</td>
<td>52.33</td>
<td>52.33</td>
<td>52.33</td>
</tr>
<tr>
<td>TEMED (µl)</td>
<td>5.67</td>
<td>5.67</td>
<td>5.67</td>
</tr>
</tbody>
</table>
The resolving gel was poured between two glass plates in a gel caster and isopropyl alcohol was added at the top to prevent formation of air bubbles. It was left for 30-45 minutes to polymerise at RT. Isopropyl alcohol was removed, the stacking gel poured and the comb inserted. It was left for 30-45 minutes to polymerise at RT. The gel (with comb kept inserted) was wrapped and stored at 4 °C and used within one week.

4.3.1.2 Protein sample preparation

Western blot was used to determine the presence or absence of the protein of interest in the cell lines that were to be used for the cell viability assays.

H69 and DMS79 cells were grown to confluence in T75 flasks (section 4.3.3). Contents of the flasks were transferred to tubes and centrifuged for 5 minutes at 42.2g and spent media discarded. Cell pellets were washed with cold PBS. Then, 250 µl of ice-cold RIPA Lysis Buffer System (prepared according to manufacturer’s instructions) were added and contents transferred to an Eppendorf® tube. The cells were lysed on a rotator for 20 minutes at 4 °C and then centrifuged for 15 minutes at 4 °C and at 12100g. The supernatant containing the desired protein lysate was removed to a new Eppendorf® tube for protein concentration determination.

Bradford assay (absorbance reading at 595 nm) was used to estimate protein concentration in the lysate. Protein samples used for western blot (45 µl maximum) were prepared by transferring a specific amount of the lysate to an Eppendorf® tube and 3X sample buffer was mixed in a volume half that of the lysate. Samples used in the same western blot run were adjusted to have the same total amount of protein. The main lysate fraction was stored at -80 °C for future applications and samples for western blot were stored at -20 °C for future analysis.

Before loading, the protein samples prepared for western blot were denatured by heating for 3 minutes at 95 °C. Heating ensures the denaturing of 2°, 3° and 4°
protein structures, while retaining 1° structure and the negative charge to move in an electric field during western blot analysis.\textsuperscript{168}

4.3.1.3 Western blotting

The ladder and the samples were loaded into the lanes of the gel in the electrophoresis tank. Empty wells were filled with sample buffer. The tank was filled with running buffer and voltage applied to carry out electrophoresis for 30 minutes at 80 V and then at 110 V for a further 50 minutes or until the marker and proteins passed through the whole gel. Then, the proteins were transferred onto the membrane for 1 hour at 180 V and 0.4 A in a transfer tank filled with an ice-cold transfer buffer.

After the transfer, the non-specific binding sites were blocked by soaking the membrane with blocking buffer for 1 hour at RT on the shaker. Then, the membrane was probed with the mouse primary antibody (GRPR (D-1) or β-Actin (C4)) (diluted in blocking buffer in a ratio of 1:1000) by shaking overnight at 4 °C. The membrane was later washed 3 times with TBS-Tween with each wash cycle being 10 minutes on the shaker. The membrane was then incubated for 1 hour at RT on the shaker with the goat anti-mouse secondary antibody labelled with HRP (diluted in blocking buffer in a ratio of 1:5000). After that, the membrane was washed with TBS-Tween as before.

Finally, the membrane was developed and bands visualised using the enhanced chemiluminescence (ECL) substrate (as per manufacturer’s instructions) and Bio-Rad imaging system.

4.3.2 Determining the concentration of peptides

The peptides synthesised contain tryptophan in their sequence can absorb UV light, attributed to the conjugated system of the indole ring. For accurate determination of the peptide concentration, a UV/Visible spectrophotometer and a 1-cm quartz cuvette were used to record the absorbance at 280 nm. The peptides were dissolved in a minimum volume of ethanol. The peptide molar concentration was determined using
the following general equation and extinction coefficients of Trp and Tyrosine (Tyr) at 280 nm:\textsuperscript{175, 176}

\[
\text{Concentration (M)} = \frac{[(\text{Absorbance} \times \text{Dilution factor})] \times (5690 \, \text{M}^{-1} \text{cm}^{-1} \times \text{number of Trp residues}) + (1280 \, \text{M}^{-1} \text{cm}^{-1} \times \text{number of Tyr residues})]}{280}
\]

In the case of this project, only the value of Trp’s extinction coefficient was of importance as the peptides lack Tyr. Although Phe has a conjugated system, its absorbance at 280 nm is so minimal; hence, it is neglected in the equation.

\textbf{4.3.3 Cell subculture}

Cells were grown in T75 or T225 flasks, depending on quantity of cells needed, at 37 °C in humidified air atmosphere of 5% CO\textsubscript{2}. They were maintained in RPMI-1640 (with L-glutamine and NaHCO\textsubscript{3}) being supplemented with FBS to make it 10% v/v FBS/RPMI-1640 (complete medium). Medium formulation specifications were obtained from ECACC.

Both, H69 and DMS79 cells are suspension cells and grow in aggregates. Splitting was done through dilution with complete medium when cells reached confluence. The dilutions used were in total volume of 12 ml and 40 ml in T75 and T225 flasks, respectively. The rest of the cells remaining, to be used for assays, were transferred to a tube and centrifuged for 5 minutes at 42.2g forming a pellet. The supernatant was decanted and cell pellet re-suspended in fresh complete medium and counted using haemocytometer and Trypan Blue\textsuperscript{172} dye.

To preserve, the cells were re-suspended in freezing medium of 5% v/v dimethylsulfoxide (DMSO)/complete medium as indicated by American Type Culture Collection (ATCC). Aliquots of 1ml of cells (3.0-9.0 x 10\textsuperscript{5} cells), suspended in freezing media, were transferred into cryogenic vials and placed inside a freezing box containing isopropyl alcohol at -80°C freezer to achieve 1°C decrease per minute to allow slow freezing. On the next day, cryogenic vials were stored in liquid N\textsubscript{2}. 
Cells were thawed by warming the cryogenic vials in a water bath at 37 °C before being transferred to a T25 flask containing 5 ml of complete medium for 2 days and incubated as before. Cells were further split into two T25 flasks for a few days to grow well. Then, cells were grown as usual in T75 or T225 flasks.

4.3.4 End-point measurement of cell viability

The measurement of cell viability, an indicator of the peptide’s cytotoxicity, was performed using the resazurin dye assay. The assay relies on an endpoint measurement in which fluorescence readings, after optimum incubation time of the dye on treated and untreated cells, are recorded and calculated as a percentage of the fluorescence of the negative control (untreated cells). Graphs were generated using GraphPad Prism 7.00 software.

4.3.4.1 Determination of optimum incubation time of resazurin dye with cells

Serial dilutions of cells above and below the intended number of cells to be used for viability assays (1 x 10⁴ cells) were made with complete medium and seeded into 96-well plates (200 µl in each well). Complete medium was used for background measurements (200 µl). The dye was prepared by dissolving resazurin sodium salt in PBS forming a solution at a concentration of 0.125 mg/ml. Resazurin solution (20 µl) was then added into each well. The plates were incubated at 37 °C throughout the experiment. The fluorescence readings were then recorded every hour up to 10 hours. The excitation wavelength was set at 530 nm and the emission wavelength at 590 nm. A calibration curve of cell number versus Arbitrary Fluorescence Units (AFU) was plotted and the line of best fit found at the optimum incubation time. The experiment was performed three independent times and in 6 replicates.

4.3.4.2 Conducting the viability assay

A few milligrams of the peptide synthesised were dissolved in a minimal volume of ethanol and its concentration calculated accurately as described above (section 4.3.2). Dilution of the stock concentration was made using 50% v/v FBS/sterile saline. Concentrations prepared ranged from 0.002 to 1.2 mM.
Cells were then seeded in 96-well plate at a seeding density of $1 \times 10^4$ cells/well/190 µl complete medium. Complete medium (190 µl) was used to determine background fluorescence. Negative controls (no treatment) were also prepared. Peptides were then added at the same time to the test wells. The volume of peptide added in each well was 10 µl resulting in a final well concentration ranging from 60 to 0.1 µM. For background fluorescence and negative control, 10 µl of 50% v/v FBS/sterile saline were added. The plates were incubated for 48 hours at 37 °C. Resazurin dye (20 µl) was then added into each well and fluorescence reading recorded after 5 hours (optimum incubation time found) and % viability calculated. All readings were corrected for background fluorescence. The experiment was performed three independent times in triplicates.

4.3.5 Assessment of apoptosis

4.3.5.1 Acridine Orange/Ethidium Bromide dual staining
The assessment of apoptosis was done using an adapted form of 96-well plate method stated in literature. Briefly, cells were seeded in a 96-well plate as described for the cell viability assays, peptides (dissolved in 50% v/v FBS/sterile saline) were added at a specific concentration on the same day as the negative controls (by adding only 50% v/v FBS/sterile saline), and then left for 48 hours incubation at 37 °C. After 48 hours, 5 µl of AO/EB mix (1:1 ratio; 100 µg/ml each dye in PBS) was added and mixed gently into each well and cells were then viewed under an inverted fluorescence microscope.

4.3.5.2 Annexin V conjugate
A quantitative measurement of apoptosis was performed using flow cytometry and Annexin V conjugate, based on detecting PS on surface of plasma membrane for cells undergoing apoptosis.

The experiment was performed according to manufacturer’s instructions. Briefly, DMS79 cells were seeded in a 12-well plate at a seeding density of $1 \times 10^5$ cells/well/1980 µl complete medium. Peptides (20 µl) (dissolved in 50% v/v
FBS/sterile saline) were added into each well resulting in a final well concentration of 2 and 6 µM and incubated for 24 hours at 37 °C. Negative controls were prepared. The contents of wells were then carefully transferred to Eppendorf® tubes and centrifuged for 5 minutes at 16.9g and media discarded. Cell pellets were washed with cold PBS. Then, cells were gently re-suspended in 100 µl annexin-binding buffer followed by the addition of 5 µl of the Annexin V conjugate (Alexa Fluor® 555) and 0.5 µl of dead cell stain (SYTOX® Blue). Tubes were incubated for 10 minutes at RT, then 400 µl of annexin-binding buffer were added and samples analysed soon after.

4.4 Results and Discussion

4.4.1 Western blot

The target protein of interest is GRPR which has a molecular weight of 43 kDa, with the glycosylated form between 70-95 kDa. For this range, 10% SDS polyacrylamide gel was appropriate to resolve the protein. A positive control had to be used to check the expression of GRPR in H69 and DMS79 cell lines. MIA PaCa-2 (pancreatic carcinoma cell line) whole cell lysate was used as a positive control. It was used by the manufacturer to test the GRPR antibody. Also, it was shown that 8-17% of human pancreatic adenocarcinomas express GRPR.20

The ladder (10 µl), protein samples (~70 µg), and MIA PaCa-2 whole cell lysate (40 µl; 100 µg) were loaded onto the lanes of the 10% gel. Proteins were resolved and then transferred onto the membrane using Bio-Rad Mini Protean® II system as described in the methods. The ladder resolved into 9 bands (180 / 130 / 100 / 70 / 55 / 40 / 35 / 25 / 15 kDa). The membrane was cut in two parts (between 70 and 55 kDa bands) to ease development and band visualisation; a part for the expected region of GRPR (70-95 kDa) and a part for the control β-Actin (43 kDa).

The primary antibodies (mouse antibodies) for GRPR and β-Actin were diluted with blocking buffer in a ratio of 1:1000. The horseradish peroxidase (HRP) conjugated secondary antibody (anti-mouse) was diluted with blocking buffer at a ratio of
1:5000. The membrane development and bands visualisation were done using ECL substrate and ChemiDoc™ MP Imaging System (Bio-Rad).

The results show (Figure 41), both H69 and DMS79 cell lines express GRPR in a similar manner to the positive control of MIA PaCa-2 whole cell lysate. The bands appeared between the markers of 70-100 kDa of the ladder which is consistent with the expected molecular weight of the glycosylated GRPR (70-95 kDa). Other groups reported the expression of GRPR in H69 cells using different methods rather than western blot, such as reverse transcriptase-polymerase chain reaction\(^{56, 58, 65}\) and RNase protection assay\(^{55}\).

**Figure 41.** Glycosylated GRPR bands obtained after western blot on H69 and DMS79 cell lines

The bands for the loading control β-Actin (43 kDa; appeared between the markers of 40-55 kDa of the ladder) for both cell lines are of similar intensity. This implies that the same amount of total protein for both cell lines was loaded onto the gel lanes (excluding the positive control).

An accurate quantitative analysis of GRPR expression would require an equal number of cells for each cell type to lyse to avoid variations of total protein obtained. In this experiment, cells of each cell type were left to grow in flasks to reach confluence and then lysed without counting the cell number. This is because the aim for this experiment was to qualitatively identify the presence of GRPR in both cell lines intended to be used in the cell viability assays. This is due to the importance of GRPR as reported in Chapter 1.
It was previously shown that 85% of patients with SCLC express GRPR. Here, for the first time the expression of GRPR in DMS79 cell line has been demonstrated.

4.4.2 Cell Viability Assays

4.4.2.1 Determination of optimum incubation time of resazurin dye with cells

The resazurin dye, when applied after treatment duration, should be incubated for an optimum incubation time to get reliable fluorescence measurement to accurately calculate % viability. This time was determined through constructing a calibration curve between variable number of cells, above and below the intended seeding density in the assays, and fluorescence readings.

4.4.2.1.1 Optimum incubation time of resazurin dye with H69 and DMS79 cells

The intended seeding density for the assays using H69 and DMS79 cells is $1 \times 10^4$ cells/well. Hence, serial dilutions of cell suspension in complete medium ranging from $1 \times 10^3$ to $1.6 \times 10^4$ cells/well/200µl were prepared and for background, 200µl/well of complete medium only was used. The doubling time for H69 cells is 65 hours and that for DMS79 cells is around 50 hours. So, $1.6 \times 10^4$ cells is an appropriate number for the maximum number of cells for a linear correlation study between AFU and number of cells. This is because the cell viability assays will be conducted for 48 hours. The optimum incubation time where there was a linear correlation between number of cells and fluorescence intensity was found to be 5 hours for both cell lines (Figure 42). The calibration curves for the 10 hours experiment for both cell lines are presented in Appendix I.
Figure 42. Calibration curves of cell number versus AFU for (a) H69 and (b) DMS79 cell lines using resazurin dye for 5 hours incubation time. Data represented with mean ± SEM, n = 3 in 6 replicates, $r^2 = 0.9995$ for both graphs.

4.4.2.2 Peptides cytotoxicity

Cell viability assays were performed as described in the methods. The number of cells seeded in each well was $1 \times 10^4$ cells. The duration for the incubation was 48 hours before resazurin dye was added, and fluorescence reading recorded after 5 hours to calculate % viability. Table 9 represents the IC$_{50}$ values for peptides 19 and 25-33 for H69 and DMS79 cell lines with standard error (SE). The dose-response curves for 19 and 25-33 are presented in Figure 43 (pages 163-165). Peptides 19 and 25-33 were tested to get an overall view of the cytotoxicity effect on cells by peptides with different N$^{\text{ind}}$-substituted D-Trp on the 4$^{\text{th}}$ residue.
Table 9. Cytotoxicity (IC$_{50}$ ± SE) of 19 and 25-33 on H69 and DMS79 cell lines after treatment for 48 hours. n = 3 in triplicates. P values; comparison to Butyl$_{\text{4th-NH}_2}$; *(<0.05); **(<0.01); ****(<0.0005).

<table>
<thead>
<tr>
<th>Peptides</th>
<th>H69 Cells</th>
<th>DMS79 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC$_{50}$ (µM) ± SE</td>
<td>IC$_{50}$ (µM) ± SE</td>
</tr>
<tr>
<td>SPG (19)</td>
<td>&gt; 60****</td>
<td>&gt; 60****</td>
</tr>
<tr>
<td>5-mer-NH$_2$ (25)</td>
<td>30.74 ± 0.30****</td>
<td>23.00 ± 2.07****</td>
</tr>
<tr>
<td>Methyl$_{\text{4th-NH}_2}$ (26)</td>
<td>3.98 ± 0.22****</td>
<td>4.03 ± 0.31\text{ns}</td>
</tr>
<tr>
<td>Ethyl$_{\text{4th-NH}_2}$ (27)</td>
<td>1.83 ± 0.03**</td>
<td>1.92 ± 0.03\text{ns}</td>
</tr>
<tr>
<td>Propyl$_{\text{4th-NH}_2}$ (28)</td>
<td>1.57 ± 0.10*</td>
<td>1.91 ± 0.03\text{ns}</td>
</tr>
<tr>
<td>Butyl$_{\text{4th-NH}_2}$ (29)</td>
<td>1.01 ± 0.02</td>
<td>1.43 ± 0.14</td>
</tr>
<tr>
<td>Pentyl$_{\text{4th-NH}_2}$ (30)</td>
<td>1.80 ± 0.10**</td>
<td>2.37 ± 0.06\text{ns}</td>
</tr>
<tr>
<td>Propargyl$_{\text{4th-NH}_2}$ (31)</td>
<td>3.30 ± 0.08****</td>
<td>3.66 ± 0.19\text{ns}</td>
</tr>
<tr>
<td>Benzyl$_{\text{4th-NH}_2}$ (32)</td>
<td>1.78 ± 0.08*</td>
<td>1.69 ± 0.06\text{ns}</td>
</tr>
<tr>
<td>tert-Prenyl$_{\text{4th-NH}_2}$ (33)</td>
<td>2.84 ± 0.14****</td>
<td>4.37 ± 0.44*</td>
</tr>
</tbody>
</table>
Figure 43. Dose-response curves for 19 and 25-33 on H69 cell line (left panel) and DMS79 cell line (right panel). Peptides treatment was for 48 hours, n = 3.

Based on the above results, peptide 29 showed the highest potency against both cell lines. Hence, further peptides were synthesised using the N<sup>ind</sup>-butyl D-Trp for optimisation. Table 10 represents the IC<sub>50</sub> values for 23, 24, 34-37 and the free amino acid (18) against H69 and DMS79 cell lines, with SE. The dose-response curves for 23, 24, 34-37 and 18 are presented in Figure 44 (pages 167-168).
Table 10. Cytotoxicity (IC$_{50}$ ± SE) of 23, 24, 34-37, and free amino acid (18) on H69 and DMS79 cell lines after treatment for 48 hours. n = 3 in triplicates. P values; comparison to Butyl$^{2nd,4th}$-NH$_2$; ****(<0.0005).

<table>
<thead>
<tr>
<th>Peptides</th>
<th>H69 Cells</th>
<th>DMS79 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butyl$^{2nd}$-NH$_2$ (34)</td>
<td>4.05 ± 0.17$^{ns}$</td>
<td>3.88 ± 0.15$^{ns}$</td>
</tr>
<tr>
<td>Butyl$^{2nd,4th}$-NH$_2$ (35)</td>
<td>0.63 ± 0.05</td>
<td>2.31 ± 0.12</td>
</tr>
<tr>
<td>Butyl$^{4th}$-OH (36)</td>
<td>&gt; 30****</td>
<td>&gt; 30****</td>
</tr>
<tr>
<td>2$^{nd}$ Butyl$^{4th}$-OH (37)</td>
<td>&gt; 30****</td>
<td>&gt; 30****</td>
</tr>
<tr>
<td>Arg-Butyl$^{3rd,5th}$-NH$_2$ (23)</td>
<td>&gt; 30****</td>
<td>&gt; 30****</td>
</tr>
<tr>
<td>Butyl$^{4th}$-SPG (24)</td>
<td>25.55 ± 2.55****</td>
<td>23.11 ± 2.60****</td>
</tr>
<tr>
<td>D-Trp(N-buty1)-OH (18)</td>
<td>&gt; 30****</td>
<td>&gt; 30****</td>
</tr>
<tr>
<td>Free amino acid</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 44. Dose-response curves for $23, 24, 34-37$ and $18$ on H69 cell line (left panel) and DMS79 cell line (right panel). Peptides treatment was for 48 hours, $n = 3$.

H69 and DMS79 are both SCLC cell lines established from the pleural fluid of a patient with SCLC.\textsuperscript{180, 181} H69 is obtained before a patient received treatment, while, DMS79 is obtained after a patient had been treated with chemotherapy (cytoxan, vincristine and methotrexate) and radiation therapy.\textsuperscript{180, 181} H69 is a classic SCLC cell line, while DMS79 is a variant SCLC cell line.\textsuperscript{16, 182} Both cell lines represent an ideal model to study how the peptides will affect classic cells (representing classic tumour) in comparison to variant cells (representing variant tumour).
In general, these in vitro viability assays showed that DMS79 cells were almost similar in sensitivity to H69. However, there were slight differences in IC$_{50}$ values for the same compound depending on cell line; most of the peptides showed higher cytotoxic activity on H69 cells than DMS79 cells. DMS79, established after chemotherapy and radiation therapy, might account for these variations. Patients with variant SCLC tumours, like the DMS79, respond poorly to chemotherapy and have short survival time compared to patients with classic tumours.$^{17,28}$ The cytotoxicity of peptides and cells sensitivity might thus vary. Although $^{19}$ showed no cytotoxic activity in tested cell lines, Waters et al.$^{65}$ showed a direct correlation between the cytotoxicity of $^{19}$ with the level of expression of neuropeptide receptors, especially GRPR, in several tumour cell lines tested. The cell lines tested were SCLC, NSCLC, colorectal, ovarian, and pancreatic cell lines.$^{65}$

The results for $^{19}$ did not show any cytotoxic activity up to 60 µM on both cell lines, while the novel $^{25}$, a closely related sequence to $^{19}$, showed better cytotoxicity. In vitro experiments, carried against H69 cell line using $^{19}$, were reported stating different IC$_{50}$ values. The IC$_{50}$ values reported were measured either by cell proliferation assays or by cell viability assays using MTT.

The IC$_{50}$ values of $^{19}$ against H69 found in literature are as follows: MacKinnon et al.$^{131}$ reported an IC$_{50}$ value of 24.5 ± 1.5 µM after 9 days in a cell proliferation assay, Sarvi et al.$^{37}$ reported an IC$_{50}$ value of 15.53 ± 1.99 µM after 72 hours using an MTT assay and Woll et al.$^{124}$ showed suppression of growth after 8 days in a cell proliferation assay using a concentration of 100 µM.

Moreira et al.$^{132}$ reported a decrease in the IC$_{50}$ value when $^{19}$ was left for a longer time with H69 cells; 261 ± 64.3 µM after 2 hours, 135 ± 15.8 µM after 24 hours, and 129 ± 7.42 µM after 48 hours, using the MTT assay. A similar decrease in IC$_{50}$ values was observed when $^{19}$ was tested on H82 cells (a variant SCLC cell line) using MTT assay by Moreira et al.$^{133}$; 148 ± 4.4 µM after 2 hours, 57.8 ± 7.2 µM after 24 hours, and 39.3 ± 19.5 µM after 48 hours. However, one trial in triplicate in
this project, was conducted to test the activity of 19 and 25 on H69 cells for 6 days, which showed similar results to the experiments conducted for 48 hours; 19 did not show any activity and 25 had an IC$_{50}$ of 35.78 µM.

Peptide 29, which bears a D-Trp(N-butyl) monomer near the C-terminal (4$^{th}$ residue), is the most potent peptide (Table 9) against both cell lines, in comparison to other peptides bearing an N$^{ind}$-substituted D-Trp on the same position.

By comparison of the peptides 25 and 33 with the DMS79 cells, the results for 33 showed that modification of the D-Trp near the C-terminal increased the potency by approximately 5 fold, in comparison to 25 which has no D-Trp modifications. Excluding 30, which bears D-Trp(N-pentyl) monomer and was less active than 29 on both cell lines, a general trend was established; as the number of carbons in a straight hydrocarbon chain increased, the potency increased. Peptide 32 which has an aromatic modification; D-Trp(N-benzyl), was more potent than 31 and 33, which have D-Trp(N-propargyl) (an alkyne) and D-Trp(N-tert-prenyl) (a branched alkene) respectively, but less potent than 29. This might suggest that increasing hydrophobicity to a certain extent increases potency.

Changing the position of the butyl modified D-Trp from near the C-terminal end to be closer to the N-terminal (2$^{nd}$ residue) (34), reduced potency by approximately 3 fold when compared to 29 on DMS79 cells. Nevertheless, this is still more cytotoxic than the unmodified peptide (25). Modifying both D-Trp residues on the 2$^{nd}$ and the 4$^{th}$ residues (35) resulted in the highest potency among all peptides against the H69 cell line. This did not apply to the DMS79 cell line, where a slight decrease in cytotoxicity of 35 was observed compared to peptide 29. This could be explained as DMS79 is a variant cell line and its sensitivity is different (Table 10).

The rest of the peptides (23, 36 and 37) showed no growth inhibition between 0.1 - 30 µM (Table 10). This indicates that the amide and Leu-NH$_2$ at the C-terminal are important for activity since the carboxylic acid peptides, 36 and 37, lost potency.
Also, the coupling of the charged amino acid (Arg) to the N-terminal of \textbf{35} (peptide \textbf{23}) made the peptide inactive. These results suggest that hydrophobicity is essential for peptide activity.

Since \textbf{29} and \textbf{35}, which have the N-butyl substituent, showed the most potent activity against H69 and DMS79 cell lines, the free amino acid D-Trp(N-butyl)-OH (\textbf{18}) was tested to check whether on its own, it was cytotoxic. On the same cell lines \textbf{18} showed no growth inhibition between 0.1 - 30 \textmu M (\textbf{Table 10}).

Furthermore, due to the effect observed on butylation, the N\textsuperscript{ind}-butyl was added to the D-Trp 4\textsuperscript{th} residue on \textbf{19}, near the C-terminal, forming \textbf{24}. Interestingly, \textbf{24} showed enhancement of cytotoxicity when compared to \textbf{19}. On both cell lines, \textbf{24} had IC\textsubscript{50} values ranging from ~23-26 \textmu M (\textbf{Table 10}) while \textbf{19} was inactive at 60 \textmu M (\textbf{Table 9}). However, \textbf{29}, which has N\textsuperscript{ind}-butyl D-Trp near its C-terminal similar to \textbf{24}, was still highly cytotoxic.

The closest literature sequences to the peptides tested here are NY3460 and NY3521. They had an IC\textsubscript{50} value of approximately 3 \textmu M after 72 hours on H69 cell line, found using \textsuperscript{3}H-thymidine incorporation assays.\textsuperscript{135} When both were used at 50 \textmu M, a concentration that completely inhibited \textsuperscript{3}H-thymidine incorporation, NY3521 suppressed growth of H69 cells and NY3460 caused a decrease in H69 cells number over a period of 12 days in cell proliferation assay.\textsuperscript{135} However, since the radiochemical \textsuperscript{3}H-thymidine is known to have drawbacks, such as cell cycle arrest induction, apoptosis, and inhibiting DNA synthesis,\textsuperscript{183, 184} these results could be an overestimate for their true cytotoxic activity and are not directly comparable to the results in this project.

To date, peptides \textbf{29} and \textbf{35} are the most potent SP peptide antagonists for the treatment of SCLC. Their cytotoxic activity was also achieved in a shorter period of time (48 hours) \textit{in vitro}, in comparison to previously reported peptides. They also act on classic (H69) and variant (DMS79) SCLC models.
4.4.3 Assessment of apoptosis

The following experiments were performed to check whether the peptides induced apoptosis to exert their cytotoxic activity on cells. The results obtained from the following assays support that these peptides induce apoptosis.

4.4.3.1 Acridine Orange/Ethidium Bromide dual staining

The assessment of apoptosis was performed as described in the methods. H69 and DMS79 cells were seeded in a 96-well plate at a density of $1 \times 10^4$ cells/well. Negative control and test samples at 0.3 µM and 6 µM final well concentrations were prepared. Apart from 19 and 25, which had IC$_{50}$ values on both cell lines above 20 µM, these concentrations (0.3 µM and 6 µM) were chosen to show the activity of 29, 33 and 35 below and above their IC$_{50}$ values. The incubation period was set at 48 hours, following which AO/EB mix was then added and the wells viewed under an inverted fluorescence microscope.

AO is taken up by viable and non-viable cells, whilst EB is taken up by cells when there is loss of plasma integrity and non-viable cells as well. AO emits green fluorescence while EB emits red/orange fluorescence when intercalated with DNA. Four stages could be detected from this assay: viable cells, early apoptotic cells, late apoptotic cells and necrotic cells. Viable and early apoptotic cells have intact plasma membranes and should thus emit green fluorescence. Although early apoptotic cells have intact plasma membranes, the chromatin condensation is initiated, which can be revealed as bright green regions. Late apoptotic cells and necrotic cells have lost their plasma membrane integrity allowing the entry of EB and causing them to emit red/orange fluorescence.

Furthermore, late apoptotic and necrotic cells can be distinguished by their size even when both can emit red/orange fluorescence. Both, necrosis and apoptosis lead to cell death. Necrosis leads to karyolysis, in which complete dissolution of the chromatin occurs, and cell swelling. On the other hand, apoptosis leads to
karyorrhexis, where the nucleus is fragmented, and cells show shrinkage. This is eventually followed by the formation of apoptotic bodies.

Such observations can clearly be seen in the following figures for assays performed on H69 and DMS79 cells treated with the different peptides. **Figure 45**, an overlay of fluorescence observed from Texas Red and FITC channels, shows green fluorescence for untreated H69 (a) and DMS79 (b) cells. This is based on AO fluorescence suggesting intact plasma membranes.

![Figure 45](image)

**Figure 45.** Untreated H69 cells (a) and DMS79 cells (b) incubated in complete media in 96-well plate for 48 hrs were stained with AO/EB (5 µl) and viewed under inverted fluorescence microscope at 10x magnification. The scale bar on each photo is 100 µm.

**Figure 46** is an overlay of fluorescence observed from Texas Red and FITC channels, to show the fluorescence observed from H69 cells after 48 hours treatment with 19, 25, 29, 33 and 35 at 0.3 µM (left panel) and 6 µM (right panel). All cells showed green fluorescence after being treated with 0.3 µM of each peptide. This is a lower concentration than their IC_{50} values and hence the majority of cells seem to maintain intact plasma membranes allowing AO entry only. The same effect was observed with 6 µM treatment of cells with peptides 19 and 25 that were previously found to be inactive at this concentration in cell viability assays (Table 9). However, 6 µM treatment with 29, 33, and 35 showed mainly red/orange fluorescence and cell shrinkage, which is consistent with apoptosis. Moreover, bright green regions were observed on cells treated with 6 µM of peptide 33, which indicates chromatin condensation of cells undergoing apoptosis. This is also observed to a lesser extent with cells treated with 6 µM of the more potent peptides (29 and 35). Similar
observations were seen with DMS79 cells when treated in the same way as H69 cells (Figure 47).

Figure 46. H69 cells incubated in complete media in 96-well plate and treated for 48 hrs with 0.3 μM (left panel) and 6 μM (right panel) of peptides 19, 25, 29, 33 and 35 were stained with AO/EB (5 μl) and viewed under inverted fluorescence microscope at 10x magnification. The scale bar on each photo is 100 μm.
Figure 47. DMS79 cells incubated in complete media in 96-well plate and treated for 48 hrs with 0.3 µM (left panel) and 6 µM (right panel) of peptides 19, 25, 29, 33 and 35 were stained with AO/EB (5 µl) and viewed under inverted fluorescence microscope at 10x magnification. The scale bar on each photo is 100 µm.
4.4.3.2 Annexin V conjugate

Apoptosis could be followed quantitatively using flow cytometry. When cells undergo apoptosis, phosphatidyl serine (PS) residues become exposed at the outer surface of the plasma membrane.\textsuperscript{172} Annexin V (AnnV) fluorescent conjugates can bind to PS, which can be measured through flow cytometric analysis.\textsuperscript{172}

In viable cells, PS is located on the inner surface of the plasma membrane and as AnnV is unable to penetrate the plasma membrane, it will not be able to bind to PS.\textsuperscript{172} AnnV is also capable of passing through the damaged membrane of necrotic dead cells and bind to the inner PS residues. These necrotic dead cells could be distinguished by the addition of dead cell stain that binds to exposed DNA, such as propidium iodide (PI).\textsuperscript{172, 188} The introduction of a dead cell stain enables the detection of four different categories: viable cells (Ann-/PI-), early apoptotic cells (Ann+/PI-), late apoptotic cells (Ann+/PI+) and necrotic cells (Ann-/PI+).\textsuperscript{185, 188}

The experiment was performed as described in the methods. DMS79 cells were seeded in a 12-well plate at a density of $1 \times 10^5$ cells/well. Two peptides, 25 and 29, were selected for the study using DMS79 cells. Peptide 29 is the most potent against
DMS79 (cell line originating from patient being treated with chemotherapeutics and radio-therapy) while 25 is the relatively less potent control peptide that has un-substituted D-Trp residues. The concentrations used for each peptide were 2 and 6 µM and compared to control (0 µM) for the untreated sample for a duration of 24 hours. These concentrations were selected to show the effect of concentration in causing apoptosis during the 24 hours period. Figure 48 is a bar-chart presentation for the flow cytometric analysis using Annexin V conjugate (Alexa Fluor® 555) and a dead cell stain (SYTOX® Blue) similar to PI. Apart from very slight increase in living cells, 25 had similar effect to the untreated cells at both concentrations (2 and 6 µM). This is an expected observation as 25 has an IC50 value of 23 µM on DMS79 (Table 9) well above the 6 µM concentration used here. However, 29 caused apoptosis in a concentration dependent manner as level of late apoptotic cells increased from 36% to 95% at 6 µM when compared to those at 2 µM. An increase of early apoptotic cells was also observed at 2 µM of 29. Living cells also decreased at both concentrations, mainly at 6 µM, when using 29. Flow cytometric dot-blot are presented in Figure 49.
Figure 48. A bar-chart presentation of the flow cytometric analysis showing the % events of live, early apoptotic, late apoptotic and necrotic cells of DMS79 using 5-mer-NH$_2$ (25) and Butyl$^{4th}$-NH$_2$ (29)
**Figure 49.** Dot blots of flow cytometric analysis for apoptosis detection using Annexin V (AnnV) and Sytox Blue (SyB) dead cell stain. DMS79 cells, untreated (a), incubated with 2 µM of 25 (b) and 29 (c) and with 6 µM of 25 (d) and 29 (e) for 24 hours in complete media in 12-well plates and analysed as described in the methods. Average percentage for each quadrant is presented with standard error (SE) (n = 3). On each dot blot, Q1 (top left) represents necrotic cells, AnnV negative (-) and SyB positive (+); Q2 (top right) represents late apoptotic cells, AnnV (+) and SyB (+); Q3 (bottom left) represents live cells, Annv (-) and SyB (-); Q4 (bottom right) represents early apoptotic cells, Ann (+) and SyB (-).

### 4.5 In vivo anti-tumour activity

One of the earlier synthesised peptides, tert-Prenyl$^{14}$-NH$_2$ (33), had its efficacy evaluated *in vivo* by our group. DMS79 xenografts model were used since this cell line is variant and obtained from patient exposed to chemotherapeutics and radiation therapy. Thus, the model is more likely to exhibit resistance to further therapy. Moreover, patients with variant SCLC tumours respond poorly to chemotherapy and have shorter survival time compared to patients with classic tumours (Chapter 1). Hence, this novel peptide was selected to check its efficacy against this model.

Ten CBA nude female mice were subcutaneously inoculated on the lower back with a suspension of $5 \times 10^6$ DMS79 cells in 50% matrigel. Once tumours reached around
250 mm$^3$, the mice were divided into two groups; half of which were treated with vehicle and half with 1.5 mg/kg peptide 33 via peri-tumoural injection three times a week. Tumours were measured 3 times a week and harvested when reaching 1000 mm$^3$. Efficacy data is shown in Figure 50 with % relative tumour volume (RTV) against time. RTV as a percentage was calculated by dividing by the tumour volume on measured days by the tumour volume on day 0 (before treatment).

Figure 50. Growth of DMS79 tumours from the start of therapy at day 0 in CBA DMS79 xenografts. Once tumours reached 250 mm$^3$ they were injected peri-tumourally with vehicle or 1.5 mg/kg of tert-Prenyl$^{4th}$-NH$_2$ (33) 3 times a week. The control and treated group each contained 5 mice. Data for the control group is shown as the mean ± SE (shaded error bands in grey). Data for the treated group is shown as individual animal data.

One of the animals (dotted line, Figure 50) in the treated group did not respond to therapy for reasons that are unclear. Excluding this single animal from the treated group, the tumour growth inhibition (TGI) (Figure 50) was calculated at day 7 as 

$$\text{TGI} (\%) = (1-T/C) \times 100,$$

where T indicates the mean tumour volume (mm$^3$) of the test group and C indicates the mean tumour volume of the vehicle-treated group. At day 7 the tumour volumes of mice treated with 33 were reduced by 30% compared with the vehicle only treated mice. This level of inhibition is significant ($p < 0.0159$), especially when considered in light of the small dosage given (1.5 mg/kg) as well as the chemo-resistant nature of the tumour model used.
The IC$_{50}$ value for 33 on DMS79 cell line was 4.37 µM. Later, Butyl$^{14}$-NH$_2$ (29) was identified to be the most potent against DMS79 cell line (IC$_{50}$ = 1.43 µM) among all synthesised peptides in this project. Thus, in the future, it would be worth studying the efficacy of 29 in vivo on the same model as it might result in better efficacy than 33.
Chapter 5
The Stability of Selected Peptides and Liposomal Formulation
Chapter 5

5. The Stability of Selected Peptides and Liposomal Formulation

5.1 Introduction

A few peptides were selected to test their stability in mouse plasma and mouse S9 liver fraction. Stability studies are useful, before any in vivo work, to access the susceptibility of the peptides to metabolism. This is to provide an indication as to whether the parent peptide would have adequate bioavailability in blood, over a specified period of time, to perform its action. The stability studies involved the use of peptides 25, 29, 33, 35 and 36. Peptide 33 was chosen as it is one of the earliest peptides synthesised and was used for in vivo tumour regression studies (Chapter 4). Peptides 29 and 35 were later studied as they were the most potent peptides. Peptide 25 was selected to compare its stability with 29, 33 and 35. This is because 25 has unmodified D-Trp residues and was used to check whether the modified D-Trp residues of 29, 33 and 35 affected stability. The selection of the de-amidated peptide (36) was based on the fact that protein de-amidation reactions are widespread in plasma. De-amidation was shown to occur with previous SP analogues (SPG (19) and SPD - Chapter 1). Hence, 36 was used to identify any other form of degradation that these sequences might be prone to.

To optimise the stability and delivery of a peptide for future in vivo work, a liposomal formulation was designed, as a proof of concept, to show the compatibility of the peptide with this delivery vehicle. Peptide 29, one of the most potent peptides, was used in this formulation. Although subcutaneous (s.c.) and intraperitoneal (i.p.) injections of the peptide are feasible, the issue of hydrophobicity might limit the amount of dose administered and/or type of vehicle used. As the peptide is hydrophobic in nature, this form of nanoparticle formulation was chosen to enhance the delivery of the peptide as it would be entrapped within the lipid bilayer of the liposomal structure, as well as in the lumen to some extent.

Liposomes have been used for cancer applications in the clinic. They can accumulate in tumours due to the enhanced permeability and retention (EPR) effect.\textsuperscript{190,191} Hence, it is good vehicle to use for SCLC for enhancing bioavailability.
5.2 Materials and Instrumentation

5.2.1 Materials

Mouse plasma was generously provided by Dr. Brian Telfer (Faculty of Biology, Medicine, and Health, University of Manchester). The S9 liver fraction (from mouse (CD-1), male, 20 mg/mL protein basis, vial of 1.0 mL), L-α-phosphatidylcholine (PC), cholesterol, Whatman Nucleopore polycarbonate membranes (0.2 and 0.1 µm pore size / 25 mm) and drain discs (25 mm) and cofactors: beta-Nictoinamide adenine dinucleotide disodium salt hydrate (NADPH), Uridine 5’-diphosphoglucuronic acid trisodium salt (UDPGA), L-Glutathione (GSH) and Adenosine 3’-phosphate 5’-phosphosulfate lithium salt hydrate (PAPS) were obtained from Sigma Aldrich. PD-10 desalting columns were obtained from GE Healthcare. Solvents and other materials were obtained from Sigma Aldrich and Fisher Scientific. Peptides 25, 29, 33, 35 and 36 synthesised previously were selected for the studies.

5.2.2 Instrumentation

The UV/VIS spectrophotometer, water bath, pipettes, centrifuge, ESIMS, rotary evaporator, freeze-dryer, incubator and RP-HPLC used are those stated in sections 2.2.2, 3.2.2 and 4.2.2. Ultrasonic water bath was obtained from Prior Laboratory Supplies Ltd. The extruder suitable for 1-10 ml scale was used and was obtained from Lipix™, Northern Lipids Inc. Zetasizer Nano Series Nano-ZS was used to determine the liposomes average diameter. Glass transition temperature (Tg) of the liposomes was measured using Differential Scanning Calorimetry (DSC 6000) obtained from PerkinElmer.

5.3 Methods

5.3.1 Stability Studies

5.3.1.1 Determining the concentration of peptides

The concentration of the peptides was determined by UV spectroscopy as described in section 4.3.2.
5.3.1.2 Conducting the stability experiment

From a known concentration of a stock peptide solution, 400 µg/ml working solution was prepared by dilution in ethanol. A 200 µl portion of the working solution was left in an Eppendorf® tube at RT to evaporate ethanol. The mouse plasma was thawed from -80 ºC, a 200 µl portion of the plasma was added to the dry peptide and incubated at 37 ºC. Then, 20 µl samples were taken at different time intervals: 0, 3, 24 and 48 hours, for analysis.

To the 20 µl sample, 80 µl of 0.1% v/v TFA/MeCN was added and left for 5 minutes prior to 1 minute sonication to precipitate soluble plasma proteins. The sample was then centrifuged for 5 minutes at 12100g. From the supernatant, 80 µl was removed and added to 120 µl of 0.1% v/v TFA/H₂O and transferred into a HPLC microvial for the sample to be analysed through a C8 column. The mobile phases used for RP-HPLC were 0.1%v/v TFA/H₂O as solution A and 0.1%v/v TFA/MeCN as solution B. A linear gradient elution was made using the method stated in section 3.3.3. The injection volume was 36 µl.

As for stability in S9 liver fraction from mouse, sampling procedure and analysis was performed as in the mouse plasma studies. The time-points were 0, 1, 2 and 3 hours. Cofactors (NADPH, UDPGA, GSH and PAPS) were added to S9 liver fractions for enzyme activation as described by Richardson et al.192 Briefly, a 200 mM Tris buffer (pH 7.4) containing 2 mM magnesium chloride was prepared. Stock solutions of the cofactors were prepared using the buffer with the following concentrations: 40 mM NADPH, 20 mM UDPGA, 2 mM GSH and 2 mg/ml PAPS. A fresh mixture of the four cofactors was prepared in a ratio of 1:1:1:1 (30 µl each) and 20 µl of the mixture was added to the sample containing the peptide and the S9 liver fraction to initiate the study. The final concentrations were approximately as follows: NADPH (1 mM), UDPGA (0.5 mM), GSH (0.05 mM) and PAPS (0.05 mg/ml).
5.3.1.3 Samples characterisation
HPLC microvials containing the samples taken at 0 and 48 hours were analysed by ESIMS.

5.3.2 Liposomal formulation
5.3.2.1 Preparation of liposomes
The preparation of the liposomes was followed as described by Aojula. This includes four main steps: preparation of the lipid film containing the peptide, lipid film hydration, extrusion and purification.

The PC and cholesterol were dissolved in 50% v/v methanol/chloroform (8 ml) at a molar ratio of 4:1 (≈ 14.4 mg PC : ≈ 1.8 mg cholesterol) in a 50 ml round bottomed flask. The peptide (150 µg - determined by UV/VIS spectrophotometer) was added to the flask, mixed and the solvents evaporated using a rotary evaporator at 40 °C for approximately 10-15 minutes to ensure a dry thin film formation. The flask was then transferred to a freeze-dryer for at least 3 hours for complete dryness.

The hydration of the film was achieved by adding PBS (4 ml) to the flask with continuous shaking in a hot water bath (55 °C) and ultrasonic water bath. This is to ensure that the film completely dislodged from the flask. Freeze-thaw cycles (5 cycles) were also performed to aid in film dislodging: the flask was frozen with liquid nitrogen and thawed with hot water bath (55 °C). The collected white cloudy suspension at this step has multi-lamellar liposomes and requires extrusion.

The extrusion was performed using the 1-10 ml extruder connected to a nitrogen gas regulator and maintained at 55 °C. The suspension was passed through two 0.2 µm and then with two 0.1 µm polycarbonate membranes placed above a drain disc. The instrument setup is presented in the reference stated before. The extrusion cycles were repeated 10 times on each pair of membranes. The collected suspension at this stage was slightly hazy with a light blue appearance and has uni-lamellar liposomes.
The suspension was then purified to collect only the liposomes and remove any free peptide, PC or cholesterol. The purification was performed on a gel filtration column using a PD-10 desalting column, following manufacturer instructions using PBS as an eluent. The volume of the liposomal formulation at the end of this process was 3.5 ml. This column represents gel filtration or size exclusion chromatography. It contains Sephadex G-25 Medium that entraps small molecular weight compounds (up to 5000 M₉) and allows large molecular weight compounds (liposomes in this case) to be separated and eluted efficiently.

5.3.2.2 Characterisation of liposomes

5.3.2.2.1 Lipid content

The lipid contents of liposomes was determined using an assay described by Stewart. This is a colorimetric method that measures the absorbance of a complex formed between the phospholipids and ammonium ferrothiocyanate. The absorbance reading is compared to a calibration curve to give the amount of lipid evaluated.

5.3.2.2.1.1 Lipid calibration curve

Ammonium ferrothiocyanate solution (0.1 M) was prepared by dissolving 27.03 g of iron (III) chloride hexahydrate and 30.4 g ammonium thiocyanate in a total volume of 1 L of distilled water. A standard solution of PC (0.5 mg/ml) was prepared in chloroform. In a 2 ml Eppendorf® tubes (5 tubes), 300 / 200 / 100 / 50 / 0 µl of PC standard solution was added and completed to 1 ml by chloroform. Ammonium ferrothiocyanate (1 ml) was then added and the mixture mixed vigorously using vortex mixer for 30 seconds. Through this, a red complex (apart from the control) was formed that is soluble in chloroform. The aqueous and the chloroform layers were separated by centrifugation for 5 minutes at 67.4g. The upper ammonium ferrothiocyanate layer was removed carefully and the bottom chloroform layer was removed and added to a cuvette and its absorbance measured at 485 nm. The experiment was repeated three independent times and a calibration curve of Absorbance versus Concentration was plotted. Graph was generated using GraphPad Prism 7.00 software.
5.3.2.2.1.2 Lipid content in the formulation
From the 3.5 ml liposomal formulation obtained after purification, 50 µl was added to the 2 ml tube, made up to 1 ml with chloroform and 1 ml of ammonium ferrothiocyanate was added and experiment was performed as above. Using the equation of the line obtained from the calibration curve and the absorbance reading of the test sample, the concentration of the lipid was found and corrected for dilution.

5.3.2.2 Peptide content
The amount of peptide in the formulation was found through RP-HPLC. A calibration curve was first constructed and served as a reference to calculate peptide content in the formulation.

5.3.2.2.2.1 Peptide calibration curve
The peptide of interest was prepared at range of concentrations from 0 - 32 µg/ml in 70% v/v H2O/MeCN (200 µl). It was analysed by RP-HPLC through a C8 column at 220 nm. A linear gradient elution was made using the method stated in section 3.3.3 using same solutions. The injection volume was 36 µl. The peak area of the peptide was recorded and calibration curve of Peak Area versus Concentration was plotted. Graph was generated using GraphPad Prism 7.00 software.

5.3.2.2.2.2 Peptide content in the formulation
From the 3.5 ml liposomal formulation obtained after purification, 120 µl was taken and added to 80 µl of 0.1% v/v TFA/MeCN. MeCN is needed to lyse the liposomes and release the peptide. The sample was analysed as above. Using the equation of the line obtained from the calibration curve and the peak area of the test sample, the concentration of the peptide was found and corrected for dilution.

5.3.2.2.3 Size and the size distribution
The average diameter of the liposomes and their polydispersity index (PDI) was measured using a Zetasizer instrument. This is done through the concept of dynamic light scattering (DLS) in which a laser beam is directed to the sample suspension
placed in a cuvette, which is kept at constant temperature. Due to the Brownian motion of the particles, the scattering intensity of the light beam fluctuates with time and this is detected through avalanche photodiode detector. The signal is then processed and analysed to get a correlation function curve to provide the average diameter and the PDI of the liposomes. The speed of the Brownian motion depends on the size of the particles and the temperature. The PDI is a measure of the uniformity of particles within a suspension. PDI values between 0.0 and 0.1 are considered a narrow monodisperse distribution, values above 0.1 are considered polydisperse and a value of zero is considered a uniform monodisperse distribution. The program was made to perform 11 runs per sample to get an average diameter and PDI. A graph was generated using OriginPro 8.5.1 software.

From the 3.5 ml liposomal formulation obtained after purification, 70 µl is added to a cuvette and inserted into the instrument. The sample was kept at 25 °C for 2 minutes before starting runs.

5.3.2.2.4 Glass transition temperature

The glass transition temperature (Tg) of the liposomal formulation was measured using a DSC 6000 instrument. Tg is the temperature at which the substance changes from a hard or glassy state into a fluid state. It is reported as a single temperature obtained from a temperature range observed in a heat flow curve. The heat flow curve is obtained by DSC method that measures the difference in heat flow to a sample and a reference, and this is presented as a function of temperature.

The instrument was first cooled to -130 °C and a stream of nitrogen gas was supplied. Then, the program was set to run between -20 °C to 40 °C with a rate of temperature change at 5 °C/minute. From the 3.5 ml liposomal formulation obtained after purification, 40 µl was added to the sample pan. Another 40 µl of PBS was added to the reference pan and both pans were kept at -20 °C for 1 minute before the program started to run. The heat flow curve obtained was analysed to evaluate Tg.
5.3.2.2.5 Encapsulation efficiency
Take into consideration the liposomal formulation volume obtained after purification, which is 3.5 ml, the encapsulation efficiency of the peptide in the formulation is presented in two ways: the percentage of peptide that remained in the final formulation from the original amount of peptide added and the amount of peptide per 1 mg of PC in the final formulation, compared to the initial value.

5.4 Results and Discussion
5.4.1 Stability studies
5.4.1.1 Stability of peptides in mouse plasma
The study was carried out for a period of 48 hours at 37 °C in which aliquots from the test sample were taken for RP-HPLC analysis at different time intervals starting at 0 (the time immediately the peptide was incubated in plasma), 3, 24 and 48 hours post-incubation as described in the methods. The experiments were repeated three independent times for each peptide.

Figure 51 presents sections of RP-HPLC chromatograms obtained for the peptides from the plasma stability study analysis. Apart from peptides 35 and 36, all the other peptides gave a peak in the RP-HPLC chromatogram, with a gradual increase over time of a new peak, attributed to a metabolite. The chromatograms for 35 showed a gradual increase of two small peaks beside the decreasing parent peptide peak, while 36 remained stable throughout the study. The chromatograms showed no other peaks apart from those presented in Figure 51 (full chromatograms are presented in Appendix I Figures 1-5).
Figure 51. Sections of RP-HPLC chromatograms obtained from the plasma stability for 25, 29, 33, 35 and 36. Chromatograms are from 0, 3, 24 and 48 hours post-incubation of peptides at 37 °C with \( t_R \) of peaks observed presented beneath the chromatograms.

The chromatograms were analysed by checking the area under peak for the parent peptide and comparing it to the area under peak for the degradation product(s) and presenting the amount left of the parent peptide as a percentage. Figure 52 is a bar-chart presentation for the relative percentage of the parent peptides left after each time interval during the test (the table is presented in Appendix I). After 48 hours of peptides incubated in plasma, the results showed that peptide 36 (inactive in cell viability assays - Chapter 4) was completely stable in plasma, with 100% of the
peptide remaining intact. The other peptides started to degrade, with 33 being the least stable with only ~36% remaining after 48 hours.

The most potent peptides; 29 and 35 (Chapter 4), had high stability in plasma as 68.5% and 81% of the peptides, respectively, remained intact even after prolonged (48 hours) exposure to plasma. The peptide which had no alkyl modifications and was weakly active in cell viability assays (25; IC\textsubscript{50} ~ 23-31 \text{\textmu}M) remained 59% intact after 48 hours.

![Figure 52. Bar-chart presentation of the relative percentage of 5-mer-NH\textsubscript{2} (25), Butyl\textsuperscript{4th}-NH\textsubscript{2} (29), tert-Prenyl\textsuperscript{4th}-NH\textsubscript{2} (33), Butyl\textsuperscript{2nd,4th}-NH\textsubscript{2} (35) and Butyl\textsuperscript{4th}-OH (36) remaining unchanged with time.](image)

Peptide 36, the de-aminated form of 29, remained completely stable. Considering the chromatograms obtained after 48 hours of plasma incubation (Figure 51), the \textit{t}_R for the metabolite of 29 (25.23 minutes) is very similar to that of 36 (25.27 minutes), but differs from the \textit{t}_R for 29, which is 24.93 minutes. This might indicate that de-amination is occurring for 29 in plasma to give 36. Peptides 25, 33 and 35 have similar structures to that of 29 and their metabolites also elute later than the parent compound suggesting their de-amination in plasma as well. Peptide 35 might have other minor pathways of degradation apart from de-amidation, due to the presence of additional minor peaks.
The amide and the de-amidated forms of the peptides differ by one Dalton: The -NH$_2$ (16 Daltons) in the amide peptide is replaced with -OH (17 Daltons) in the de-amidated (carboxylic acid) peptide. Hence, MS analysis for the RP-HPLC microvial samples at 0 and 48 hours could detect the difference between these peptides.

The MS spectrum for the 0 hour sample obtained from the stability study of 36 showed the most abundant ion for the parent amide compound at $m/z$: 868.5 [M+H]$^+$ (calculated [M+H]$^+$ is 868.4756) (Figure 53). The same predominant ion was observed for the 48 hour sample (Figure 53) as 36 was completely stable in plasma.
Figure 53. ESIMS spectra for the 0 and 48 hours samples obtained from the stability study of 36 in mouse plasma. The calculated [M+H]$^+$ for 36 is 868.4756 and the most abundant ion observed at 0 and 48 hours samples was 868.5.
The MS spectrum for the 0 hour sample obtained from the stability study of 33 showed the most abundant ion for the parent compound at $m/z$: 879.5 [M+H]$^+$ (calculated [M+H]$^+$ is 879.4916) (Figure 54). On the other hand, the most abundant ion observed for the 48 hours sample was 880.5 [M+H]$^+$, which resembles the calculated [M+H]$^+$ for the de-amidated form of 33 (880.4756) (Figure 54).
Figure 54. ESIMS spectra for the 0 and 48 hours samples obtained from the stability study of 33 in mouse plasma. The calculated [M+H]$^+$ for 33 is 879.4916 and the most abundant ion observed at 0 hour sample was 879.5. The calculated [M+H]$^+$ for the de-amidated form of 33 is 880.4756 and the most abundant ion observed at 48 hours sample was 880.5.
Similar observations were shown for the samples obtained from the stability study of 25. The most abundant ion observed for the 0 hour sample was for the parent compound (25) at m/z: 811.5 [M+H]⁺ (calculated [M+H]⁺ is 811.4290) (Appendix I Figure 6). The most abundant ion observed for the 48 hours sample was 812.5 [M+H]⁺, which resembled the calculated [M+H]⁺ for the de-amidated form of 25 (812.4130) (Appendix I Figure 6).

Although the degradation of 29 was observed in mouse plasma, the MS spectra for the 0 and 48 hours samples showed the same predominant ion for the parent peptide at m/z: 867.5 (calculated [M+H]⁺ is 867.4916) (Appendix I Figure 7). Similar observations were shown for 35 as the predominant ion observed for the 0 and 48 hours sample was 923.6, resembling the calculated [M+H]⁺ of the parent compound (923.5542) (Appendix I Figure 8). This indicates that the major components remaining are the native butylated and amidated sequences. This can be explained since peptides 29 and 35 showed relatively higher stability than peptides 25 and 33.

A further experiment was done to confirm de-amidation by using 29 and its de-amidated form 36. Both peptides were mixed and analysed by RP-HPLC to check their t_R first and then co-incubated in mouse plasma for 48 hours. From a working concentration of 400 µg/ml in ethanol for both peptides, 200 µl of 29 and 80 µl of 36 were mixed. Then, 20 µl of the mixture was diluted as in plasma stability studies for studying their elution. The remaining mixture was left to dry and then incubated in plasma. Through this, the relative amount of the de-amidated form was kept less than that of the amide form in order to compare the results.

**Figure 55** showed that the de-amidated compound eluted later than the amide compound since it has a smaller peak as a result of the lower quantity added from the beginning (full chromatogram see Appendix I Figure 9). These results allow to assign t_R to the free carboxylic acid (36) and the amidated (29) forms and confirm what was observed in the plasma stability studies performed before, with regards to de-amidation. The t_R are slightly different as the samples were already dissolved in
ethanol when tested on RP-HPLC, which might affected the elution. This unambiguously shows that the de-amidated product for the peptides of interest elutes later than the parent compound.

**Butyl<sup>4th</sup>-NH<sub>2</sub> (29) and lower amount of Butyl<sup>4th</sup>-OH (36)**

$t_R$ (mins) 24.66 / 24.97

**Figure 55.** Section of the RP-HPLC chromatogram for the analysis of the mixture of 29 and lower amount of 36

**Figure 56** presents sections of the RP-HPLC chromatograms for the mixture of both peptides (29 and 36) after being incubated in plasma. These profiles are similar to those obtained for the degradation of 29 with similar $t_R$ (**Figure 51**). Data shows the de-amidated form is increasing while the parent amide peptide is decreasing with time, as expected. The relative percentage of each compound at their specific $t_R$ are presented beneath the chromatograms (full chromatograms see Appendix I Figure 10).
Butyl\textsuperscript{4th-}NH\textsubscript{2} (29) and lower amount of Butyl\textsuperscript{4th-}OH (36)

Figure 56. RP-HPLC chromatograms for the plasma stability study performed on the mixture containing 29 and a lower amount of 36.

The MS spectra, Figure 57, support the RP-HPLC results as a predominant ion for the 48 hours sample was observed at \textit{m/z}: 868.5, corresponding to the de-amidated form (calculated [M+H]\textsuperscript{+} is 868.4756). This is an expected observation as the percentage of the de-amidated compound rose from \textasciitilde30% at 0 hour to \textasciitilde61% at 48 hours (Figure 56). The predominant ion at 0 hour was at \textit{m/z}: 867.5 that represents the amide compound (calculated [M+H]\textsuperscript{+} is 867.4916) since its percentage was \textasciitilde70% as intentionally prepared at the start.
Figure 57. ESIMS spectra for the 0 and 48 hours samples obtained from the stability study for the mixture of 29 and 36 in mouse plasma. The calculated [M+H]$^+$ for 29 is 867.4916 and the most abundant ion observed at 0 hour sample was 867.5. The calculated [M+H]$^+$ for the de-amidated form of 29 (36) is 868.4756 and the most abundant ion observed at 48 hours sample was 868.5.
The RP-HPLC chromatograms and MS spectra supports that de-amidation is the likely product of metabolism of the peptides in plasma. There was no other form of degradation in plasma as there were no other peaks observed in the chromatograms. With the exception of 33, it is suggested that N⁰-alkyl modifications of D-Trp residues in the peptide sequences increases peptides stability with regards to metabolism in plasma. Also, dual N⁰-alkyl modifications on the peptide sequence (35) further enhanced the stability of the peptide. This is seen as the unmodified peptide (25) was less stable than 29 and 35 that carry one and two butyl group modifications, respectively. Furthermore, apart from 36, the most stable peptides, 29 and 35, are also the most potent peptides as observed in cell viability assays. The ranking order of peptide stability from both RP-HPLC and MS data is 36 > 35 > 29 > 25 > 33.

As previously mentioned (Chapter 1 - Section 1.4.2.4), the stabilities of SPG (19), SPD and [D-Arg¹,D-Trp⁵,⁷,⁹,D-Leu¹⁰,Leu¹¹]SP-OH were evaluated in vitro and in vivo and showed that the latter two analogues are more stable than 19. The results of this study show that the peptides synthesised in this project gave a further enhancement to stability than the three analogues just mentioned.

One of the metabolites being identified for 19 and SPD was their de-amidated forms. Thus, de-amidation is considered a common stability issue in these SP related analogues. The elution of the de-amidated product might vary according to the sequence and conformation of the peptides. RP-HPLC analysis of the de-amidated form of SPD tested previously,141 showed that it eluted later than the parent amidated peptide (SPD) as in the case observed here for peptides 25, 29, 33 and 35. On the other hand, RP-HPLC analysis for the de-amidated form of 19 showed that it eluted earlier than the amidated parent peptide (19).139,140

Peptide de-amidation could be a result of hydrolyses catalyzed by serine carboxypeptidases enzymes. Serine carboxypeptidases are capable of de-amidating peptides (de-amidase activity) and the removal of a C-terminal amino acid along
with the amide (carboxypeptidase activity). It has been identified that these types of enzymes are responsible for the metabolism of 19 and it is possible to have a similar situation for SPD. The only observed form of metabolism in this study was de-amidation; this could be as a result of the nature of the sequences, which could change enzymes selectivity. The C-terminal end comprises of L amino acid and also may have a higher degree of freedom since it is not part of the peptide backbone. This may make it the only labile location susceptible to enzymatic action.

5.4.1.2 Peptides stability in S9 liver fraction from mouse
S9 liver fraction would give further information regarding the stability of the peptides since the preparation consists of Phase I-microsomal (oxidation, reduction or hydrolysis) and Phase II-cytosolic (conjugation) metabolising enzymes. These might further accelerate degradation. The unmodified peptide 25 was used along with the modified most potent peptides (29 and 35). The experiment was performed for a period of 3 hours as described in the methods and repeated three independent times. Figure 58 shows both peptides (29 and 35) were completely stable, while 25 showed slight degradation which could be de-amidation, as described before for the mouse plasma study. Peptide 25 was almost 95% stable after 3 hours incubation in S9 liver fraction. The full RP-HPLC chromatograms are presented in Figures 11-13 in Appendix I.
Figure 58. Sections of RP-HPLC chromatograms obtained from the S9 liver fraction for the stability of peptides 25, 29 and 35. Chromatograms are from 0, 1, 2 and 3 hours post-incubation of peptides at 37 °C with $t_R$ of peaks observed presented beneath the chromatograms. The % of remaining parent peptide for 25 is presented beneath the chromatograms.

In summary, the most potent peptides, 29 and 35, also turned out to be the most stable peptides synthesised in this study. They are also shown to be more stable than the previously tested SP analogues (in vitro), both in plasma and S9 liver fraction from mouse.
5.4.2 Liposomal formulation

Peptide 29 was chosen for the liposomal formulation as being one of the most potent peptides. The formulation was prepared and characterised as described in the methods section. The peptide would mainly be expected to reside within the hydrophobic phospholipid bilayer due to its low aqueous solubility, while entrapment within the hydrophilic lumen of the liposomes would be limited.

The calibration curves for 29 (Figure 59) and PC (Figure 60) were completed as discussed in the methods. The equation of the line of best fit obtained from each curve was used to obtain the amounts of material in the tested samples.

Figure 59. The calibration curve for 29 obtained from RP-HPLC analysis through C8 column at 220 nm. The equation of the line is \[ y = 117899x + 21872 \]. Error bars are too small to be observed, \( n = 3, r^2 = 0.9961 \).
The method was found to be successful in encapsulating 29 within the liposomes. This was reconfirmed by the preparation of three formulations to check the reproducibility of the method. In 3.5 ml final volume of liposomal formulation, the concentration of 29 was 29.3 µM ± 1.9 (88.9 µg ± 5.6 from 150 µg originally added; ≈ 60% encapsulation), the average diameter of liposomes was 114 ± 1 nm and the Tg was found to be 11.5 °C ± 0.5. The value obtained for Tg is consistent with what can be expected; PC by itself has a Tg of -15 °C and with the presence of cholesterol and the peptide, the lipid bilayer would become more rigid and needs more heat energy to become fluid. Hence, a rise in the Tg value was observed.

Using one of the formulations as an example, the encapsulation efficiency of the peptide was found to be 66.1% (99.2 µg from 150 µg originally; 32.7 µM), which represents 8.7 µg peptide/1 mg PC (total PC in 3.5 ml was 11.4 mg). These results are related to the 3.5 ml liposomal fraction obtained after purification. The average diameter of the liposomes in this formulation was 112 nm with PDI = 0.052 (Figure 61). The Tg was found to be 12.08 °C.
The hydrophobicity of 29 could compromise its delivery to the site of the tumour. However, to overcome this obstacle, liposomal formulation will allow intravenous (i.v.) administration via tail vein for potential tumour regression studies. Furthermore, the stability of 29 would be optimised as it would be less exposed to metabolising enzymes. The alternative of making the peptide less hydrophobic by introducing charged amino acid (Arg) or removing hydrophobic amino acid (Leu) is a less attractive approach, as such analogues resulted in loss of the cytotoxic activity (Chapter 4 - Table 10).

Moreover, the liposomal formulation can make use of the EPR effect observed in tumours.191 Most solid tumours, including SCLC, are characterised by having extensive and yet leaky vasculature and enhanced vascular permeability. These arise due to angiogenesis and other permeability-enhancing factors, such as BK, VEGF, nitric oxide and matrix metalloproteinases, and collectively contribute to the EPR effect.191 The EPR effect enables the entrapment of large biocompatible molecules, such as macromolecular drugs and nanoparticles (e.g. liposomes), in tumours for a prolonged period of time in comparison to free drugs which diffuses back to blood circulation. Previously, it has been shown that the tissue distribution of 19, following

Figure 61. Size distribution of liposomal formulation containing 29. Obtained from 11 runs of 70 µl sample, average size is 112 nm and PDI is 0.052.
its i.p. injection into mice bearing H69 xenografts, is the result of vascular permeability rather than passive diffusion or tissue blood flow.\textsuperscript{140} This was elucidated as the H69 xenograft and organs with leaky vessels, such as liver, spleen and kidneys, accumulated most of 19, whereas organs with least permeable membranes, such as lung, heart and brain, accumulated the lowest levels of 19. Hence, the delivery, stability and efficacy of 29 are expected to be optimised with the use of liposomal formulations.
Chapter 6
Summary, Conclusions and Future Work
6. Summary, Conclusions and Future Work

6.1 Summary and conclusions

This project aimed to design, synthesise and biological evaluate novel SP analogues (peptides) for the treatment of SCLC. There were 15 novel peptides synthesised and their cytotoxicity was evaluated on two SCLC cell lines: H69, a chemo-naive cell line from a patient before treatment, and DMS79, a cell line obtained from a patient after treatment with chemotherapeutics and radiation therapy. The stability of selected peptides was evaluated in plasma and S9 liver fraction from mouse and one of the most potent peptides was shown to be compatible with a liposomal formulation. In addition, one analogue was also evaluated in vivo using SCLC xenografts in mice.

There were two series of analogues synthesised in this project based on a novel lead pentapeptide (DMePhe-DTrp-Phe-DTrp-Leu-NH₂) (25). The chemical strategy adopted was to modify the D-Trp residue(s) side chain within their sequence in comparison to 25. Specifically, modifications applied were the addition of different substituents on the nitrogen atom of D-Trp’s indole side chain (N\textsuperscript{ind}).

The first series consisted of peptides with D-Trp on the 4\textsuperscript{th} position modified with various substituents; methyl, ethyl, propyl, butyl, pentyl, propargyl, benzyl and tert-prenyl. The 4\textsuperscript{th} position was chosen as previously related SP analogues showed that the C-terminal was prone to degradation rather than the N-terminal. Also, the metabolites, which arose from C-terminal degradation, were not as active as the parent peptides. Hence, applying modifications near the C-terminal were expected to enhance the peptides’ stability and cytotoxic activity. SPG (Arg-DTrp-MePhe-DTrp-Leu-Met-NH₂) (19), SP analogue mentioned in the literature that had entered Phase I clinical trials, was also synthesised to compare its activity with the novel peptides.

Interestingly, 25 was more potent than 19 as its IC\textsubscript{50} values were ~23 µM (H69) and ~31 µM (DMS79), in contrast to SPG that did not show any cytotoxic activity at 60 µM on both cell lines. Moreover, the substituents caused a massive enhancement of
cytotoxic activity of the peptides compared to 25, as their IC\textsubscript{50} values were <5 µM. The most potent peptide of this series was that with the N-butyl substituent (29) (IC\textsubscript{50} ~1 µM on H69 and ~1.4 µM on DMS79). Compared to 25, these results showed that the substituents on D-Trp’s N\textsuperscript{ind} played a major role in enhancing the cytotoxicity of the peptide. The observed effects may be a result of altered ligand-receptor binding. This can be expected from the loss of hydrogen bonding and gain of hydrophobic interactions as the indole-H was substituted with hydrophobic groups that had no hydrogen bond acceptor or donor properties. Thus, the peptides may change the conformation of the receptor and inhibit its signalling pathways that are usually initiated by growth factors.

A second series of peptides were synthesised to optimise 29 using the D-Trp with butyl substituent; D-Trp(N-butyl). The incorporation of D-Trp(N-butyl) in the 2\textsuperscript{nd} position instead of the 4\textsuperscript{th} position decreased the peptide’s cytotoxic activity; IC\textsubscript{50} ~3.9 µM (H69) and ~4.1 µM (DMS79). Hence, the 4\textsuperscript{th} position appears to be the preferred site for modification rather than the 2\textsuperscript{nd} position for enhancing cytotoxicity. However, there was a slight increase in cytotoxic activity when incorporating D-Trp(N-butyl) on both the 2\textsuperscript{nd} and 4\textsuperscript{th} positions (35) as observed with the H69 cell line (IC\textsubscript{50} ~0.6 µM), but with a slightly lower cytotoxicity on DMS79 cells (IC\textsubscript{50} ~2.3 µM). The fact that modifying the peptide’s 2\textsuperscript{nd} position showed improvement in comparison to 25 and modifying 2\textsuperscript{nd} and 4\textsuperscript{th} positions further enhanced activity, again suggests changes of ligand-receptor binding due to the N-butyl substituents.

The results implied that hydrophobicity and hydrophobic interactions are important for the cytotoxicity of the peptides. This is substantiated as 25 consisted of only hydrophobic amino acids and showed better activity than 19 that has both hydrophobic amino acids and a charged amino acid (Arg). Furthermore, any modifications applied to increase hydrophilicity made the peptides inactive even at 30 µM. This was further confirmed through modifying 29 by changing its C-terminal amide into a carboxylic acid or the removal of the hydrophobic Leu-NH\textsubscript{2}, and the addition of Arg to 35. Increasing the hydrophobicity of 19 by the incorporation of D-
Trp(N-butyl) in its 4\textsuperscript{th} position (24), enhanced the peptide’s cytotoxicity with IC\textsubscript{50} values of \(\sim 23\ \mu\text{M}\) (H69) and \(\sim 26\ \mu\text{M}\) (DMS79).

The cytotoxic effect of the peptides seems to proceed with the initiation of apoptosis in cells, as elucidated from two fluorescent techniques. When cytotoxic peptides (29, 33 and 35) were used at a concentration of 6 \(\mu\text{M}\), higher than their IC\textsubscript{50} values (0.63 to 4.37 \(\mu\text{M}\)), red/orange emission and shrinkage of cells were observed via dual AO/EB staining. These observations are characteristic for apoptosis. In contrast, green emission and normal cells size were observed using 29, 33 and 35 at a concentration of 0.3 \(\mu\text{M}\), below their IC\textsubscript{50} values, or using the relatively inactive peptides (19 and 25) at 0.3 and 6 \(\mu\text{M}\). Apoptotic cells were quantified via flow cytometry using Ann V conjugate, which binds to PS residues that become exposed at the surface of cells undergoing apoptosis. Flow cytometric analysis of 29 on the DMS79 cell line (IC\textsubscript{50} = 1.43 \(\mu\text{M}\)) showed that the level of late apoptotic cells rose from 36\% at 2 \(\mu\text{M}\) to 96\% at 6 \(\mu\text{M}\), compared to 25 that exhibited no effect using the same concentrations.

Apart from the ability of the N-butyl substituents to enhance the cytotoxicity of the lead peptide, they improved the stability of the peptides as well. This could be as a result of the peptides structures hindering their binding to the metabolising enzymes. This was evident as the peptides modified with D-Trp(N-butyl) (29 and 35) were more stable than the unmodified peptide 25 in plasma and S9 liver fraction from mouse. Exposed to neat mouse plasma for 48 hours, 29 and 35 remained intact by 68.5\% and 81.0\%, respectively, compared to 59.0\% for 25, evaluated by RP-HPLC. Complete metabolic stability of 29 and 35 was also observed after 3 hours incubation in S9 liver fraction from mouse, in contrast to 25 which was 94.9\% intact. In addition, the only form of degradation affecting these peptides was de-amidation, as elucidated by RP-HPLC and MS, while the previously mentioned peptides were additionally susceptible to oxidation and amino acid cleavage. Also, the results showed that the most potent peptides are more stable than the previously mentioned peptides in the literature.
Although the hydrophobicity of the peptides was a major contribu-
tor to cytotoxicity
and stability, it negatively impacted on their aqueous solubility. This could be a
significant issue for in vivo delivery. Increasing the hydrophilicity of the peptides
caused loss of activity. Hence, a liposomal formulation was designed to overcome
this shortfall. One of the most potent peptides (29) was compatible with the
liposomal formulation. This was shown to be feasible by incorporating 29, mainly in
the lipid bilayer of liposomes, to potentially serve as a peptide delivery vehicle for
future in vivo studies.

6.2 Future work
As the novel peptides synthesised in this project were cytotoxic against both cell
lines (H69 and DMS79), in which DMS79 was obtained from a patient after
treatment, it is possible that they may have the potential to target chemo-resistant
cells. Moreover, the efficacy of 33 was separately evaluated in vivo using DMS79
xenografts by our group.\textsuperscript{189} A low dose (1.5 mg/kg) was found to reduce tumour
growth by 30\% (p < 0.05) at day 7, relative to controls. Higher doses could not be
used due to limited aqueous solubility. However, the low concentration of 33 used
for our in vivo work was much lower than the concentrations used for the previously
mentioned SP analogues (25-45 mg/kg) (Chapter 1). Thus, 33 is more efficacious
than the previous analogues and the more potent peptides identified in this project
(29 and 35) might result in still better efficacy in vivo.

It was also presented that 29 and 35 are more stable in mouse plasma than 33.
Peptide 33 remained 35.9\% intact after 48 hours incubation compared to 68.5\% and
81.0\% for 29 and 35, respectively. The feasibility of incorporating 29 into liposomes
to enhance delivery and solubility was demonstrated in this project. Collectively,
these results show the possibility to use a liposomal preparation of 29 for future in
vivo efficacy testing against SCLC to additionally take advantage of the EPR effect.

Based on the results of this project, further developments could be envisaged to fine
tune more peptide candidates for SCLC treatment. For example, modifications on the
indole group of D-Trp could be at different positions on the carbon atoms instead of the nitrogen atom. The synthesis of \( N^{\text{ind}} \)-tert-prenylated D-Trp was performed using palladium-catalyst. Using the same catalyst and different reaction conditions, modifications on the carbon atoms of D-Trp could also be possible. The \( C^{\text{ind}} \)-modified D-Trp residues could be incorporated into peptides for further cytotoxic evaluations.

This project shows that the active peptides have a unique sequence comprising DMePhe-DTrp(N-R)-Phe-DTrp(N-R)-Leu-NH\(_2\), which is critical to its function. It shows that any amino acid deletion (37; Leu removal) or addition (23; Arg addition) renders the peptide inactive against SCLC. Hence, the available options for further investigations could be the conversions of L amino acids (Phe and Leu) to their D isomer or changing the positions of amino acids within the peptide sequence.

Finally, apart from SCLC, GRP is shown to be a mitogen in cancer cells, such as prostate, breast, gastric, pancreatic and colorectal cancers and there is an overexpression of GRPR in these cancers. SP analogues synthesised in this project, which are potentially targeting GRPR amongst other receptors, could be screened to test their cytotoxicity against these cancer cells. This would greatly expand the spectrum activity profile for the novel SP analogues developed in this project.
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References


Appendix I. Resazurin Calibration Curves and RP-HPLC Chromatograms/MS Spectra of Stability Studies
Resazurin Calibration Curves

1. H69 Cells

The experiment was conducted 3 independent times in 6 replicates. Error bars are removed to clearly present the graph. The value for $r^2$ after 1 hour was 0.9404 and after 10 hours was 0.9984. The best fit line was after 5 hours with $r^2$ of 0.9995.
2. DMS79 Cells

The experiment was conducted 3 independent times in 6 replicates. Error bars are removed to clearly present the graph. The value for $r^2$ after 1 hour was 0.9928 and after 10 hours was 0.98. The best fit line was after 5 hours with $r^2$ of 0.9995.
Stability Studies

Table 1. Relative % (± SE) of 25, 29, 33, 35 and 36 remained after each time interval during mouse plasma stability studies

<table>
<thead>
<tr>
<th></th>
<th>0 hr</th>
<th>3 hrs</th>
<th>24 hrs</th>
<th>48 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-mer-NH₂ (25)</td>
<td>100 ± zero</td>
<td>98.03 ± 0.56</td>
<td>80.43 ± 2.33</td>
<td>59.00 ± 5.11</td>
</tr>
<tr>
<td>Butyl¹⁴⁻NH₂ (29)</td>
<td>100 ± zero</td>
<td>94.30 ± 0.20</td>
<td>80.30 ± 0.32</td>
<td>68.47 ± 0.23</td>
</tr>
<tr>
<td>tert-Preny¹⁴⁻NH₂ (33)</td>
<td>100 ± zero</td>
<td>87.77 ± 1.47</td>
<td>57.73 ± 0.83</td>
<td>35.87 ± 1.74</td>
</tr>
<tr>
<td>Butyl²⁺¹⁴⁻NH₂ (35)</td>
<td>100 ± zero</td>
<td>98.67 ± 0.69</td>
<td>89.73 ± 0.68</td>
<td>81.00 ± 2.25</td>
</tr>
<tr>
<td>Butyl²⁻⁴⁻OH (36)</td>
<td>100 ± zero</td>
<td>100 ± zero</td>
<td>100 ± zero</td>
<td>100 ± zero</td>
</tr>
</tbody>
</table>

Peptide 25 (5-mer-NH₂)

Figure 1. RP-HPLC chromatograms at 0/3/24/48 hours for 25 in mouse plasma stability studies
Peptide 29 (Butyl\textsuperscript{4th}-NH\textsubscript{2})

Figure 2. RP-HPLC chromatograms at 0/3/24/48 hours for 29 in mouse plasma stability studies

Peptide 33 (\textit{tert}-Prenyl\textsuperscript{4th}-NH\textsubscript{2})

Figure 3. RP-HPLC chromatograms at 0/3/24/48 hours for 33 in mouse plasma stability studies
Peptide 35 (Butyl^{2nd,4th}-NH_2)

Figure 4. RP-HPLC chromatograms at 0/3/24/48 hours for 35 in mouse plasma stability studies

Peptide 36 (Butyl^{4th}-OH)

Figure 5. RP-HPLC chromatograms at 0/3/24/48 hours for 36 in mouse plasma stability studies
Figure 6. ESIMS spectra for the 0 and 48 hours samples obtained from the stability study of 25 in mouse plasma. The calculated [M+H]^+ for 25 is 811.4290 and the most abundant ion observed at 0 hour sample was 811.5. The calculated [M+H]^+ for the de-amidated form of 25 is 812.4130 and the most abundant ion observed at 48 hours sample was 812.5.
Figure 7. ESIMS spectra for the 0 and 48 hours samples obtained from the stability study of 29 in mouse plasma. The calculated [M+H]$^+$ for 29 is 867.4916 and the most abundant ion observed at 0 and 48 hours samples was 867.5.
Figure 8. ESIMS spectra for the 0 and 48 hours samples obtained from the stability study of 35 in mouse plasma. The calculated [M+H]^+ for 35 is 923.5542 and the most abundant ion observed at 0 and 48 hours samples was 923.6.
The mixture of 29 (Butyl$^{4\text{th}}$-NH$_2$) and a lower concentration of 36 (Butyl$^{4\text{th}}$-OH)

Figure 9. RP-HPLC chromatogram for the mixture of 29 and 36

The mixture of 29 (Butyl$^{4\text{th}}$-NH$_2$) and a lower concentration of 36 (Butyl$^{4\text{th}}$-OH)

Figure 10. RP-HPLC chromatograms at 0/3/24/48 hours for the mixture of 29 and 36 in mouse plasma stability studies
Peptide 25 (5-mer-NH$_2$)

Figure 11. RP-HPLC chromatograms at 0/1/2/3 hours for 25 in S9 liver fraction from mouse stability studies

Peptide 29 (Butyl$^{4\text{th}}$-NH$_2$)

Figure 12. RP-HPLC chromatograms at 0/1/2/3 hours for 29 in S9 liver fraction from mouse stability studies
Peptide 35 (Butyl$^{2\text{nd},4\text{th}}$,NH$_2$)

Figure 13. RP-HPLC chromatograms at 0/1/2/3 hours for 35 in S9 liver fraction from mouse stability studies
Appendix II. Publications
MedChemComm Front Cover. The image consists of a 3D structure for the tert-Prenyl$^{14}$-NH$_2$ (33) peptide and the background is the result obtained from AO/EB dual staining experiment using 33 on DMS79 cell line at 6 µM.
N-tert-Prenylation of the indole ring improves the cytotoxicity of a Short antagonist G analogue against small cell lung cancer\textsuperscript{†}\textsuperscript{†}

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Natural prenylated indoles have been proposed as potential anticancer agents. To exploit this discovery for developing new peptide therapeutics, we report the first studies whereby incorporation of prenylated indoles into primary sequences has been achieved. We developed a route to synthesise N-\textsuperscript{\textit{tert}}-farnesyl-protected tryptophan derivatives in which the prenyl group is linked to the N-\textit{tert}-colona by using trifluoroacetic acid-promoted C-H functionalisation of 2-ethyl-3-butene. Based on the Substance P antagonist G SP65, a well-known Small Cell Lung Cancer (SCLC) anticancer agent, we designed a new prenyl peptide sequence to include a prenyl moiety on one of the tryptophan residues. The N-tert-prenylated tryptophan analogue was assembled into the pantomonic peptide using standard solid phase peptide synthesis or liquid phase synthesis by fragment coupling. In vitro screening showed that the N-\textit{tert}-prenylation of the indole ring on the tryptophan residue located near the \textit{C}-terminal of the pre peptide enhanced the cytotoxicity against HE9 (IC\textsubscript{50} = 2.84 ± 0.14 \mu M) and DM29 (IC\textsubscript{50} = 4.37 ± 0.44 \mu M) SCLC cell lines when compared with the unmodified pantomonic peptide (IC\textsubscript{50} = 30.74 ± 0.30 \mu M and DM29 (IC\textsubscript{50} = 23.00 ± 2.07 \mu M or the parent SP65 sequence (IC\textsubscript{50} = 30 \mu M) cell lines. SCLC almost inevitably relapses with therapy-resistant disease. The DM29 cell line was established from a patient following treatment with a number of chemotherapeutics (cytarabine, vincristine and methotrexate) and radiation therapy. Treatment DM29 tumour-bearing nude mice provided a human xenograft model of drug resistance to test the efficacy of the prenylated peptide. A low dose (1.5 mg kg\textsuperscript{-1}) of the prenylated peptide was found to reduce tumour growth by ≤ 80% (P < 0.05) at day 7 relative to the control group receiving vehicle only. We conclude that the availability of the Fmoc-Tip\textsubscript{\textit{tert}}-prenyl-OMe amino acid facilitates the synthesis of prenylated tryptophan-containing peptides to explore their therapeutic potential.

Introduction

One in five cases of lung cancer is attributed to small cell lung cancer (SCLC). SCLC is extremely aggressive and metastasises rapidly to the brain, liver and bones.\textsuperscript{1} Removal of SCLC tumours by surgery is generally ruled out, leaving chemotherapy and radiotherapy as alternative treatment options. However, as available cytotoxic agents currently lack the necessary potency for effective long-term treatment, the benefits from chemotherapy are short-lived with only around 5% survival rate five years post diagnosis.\textsuperscript{2,3} Mainstream treatment consists largely of cisplatin or carboplatin with etoposide and radiotherapy.\textsuperscript{4-7} Toxicity and rapid emergence of resistance to chemotherapy and radiotherapy occurs, limiting the success of further treatment cycles.\textsuperscript{2,5} Over the last three decades, there has been minimal success in developing better chemotherapies for SCLC. To address many of these challenges, it is logical to refocus attention on developing agents that are both potent and broad-spectrum, hitting multi-targets.

SCLC is a neuroendocrine cancer that secretes a range of neuropeptides which act, through autocrine and paracrine loops, as growth factors to stimulate and sustain the proliferation of tumour cells.\textsuperscript{8-10} Among these are the calcium-mobilising neuropeptides, such as the gastrin-releasing
peptide (GRP), bradykinin, cholecystokinin, neurotensin and vasoressin. Many peptide antagonists have been designed based on targeting a specific receptor of a particular growth factor. However, synthetic peptide analogues, which can effectively intercept multiple of neuropeptide loops, offer better hope for wider spectrum antagonism. One well-studied example is a hexa-peptide analogue named Substance P Antagonist C (SPC), comprising the sequence Arg-e-Trp-\text{NMePhe}-e-Trp-Leu-Met-NH₂.\textsuperscript{13,14} It has a favourable inhibitory effect on experimental SCLC models, both in vitro and in vivo, and can antagonise the action of multiple neuropeptide agonists. Importantly, SPC also showed dose-dependent inhibition of human SCLC cell lines in vitro in the range 24.5–38.5 μM (ref. 14) and significantly impaired xenograft tumour growth in nude mice.\textsuperscript{15,16} In addition to acting as an antagonist to block cell growth, SPC was also able to sensitize cells to chemotherapy and induce apoptosis.\textsuperscript{17}

Recognising the anti-proliferative action together with other preclinical evidence supporting broad-spectrum neuropeptide antagonism prompted a phase I clinical trial with SPC,\textsuperscript{18} the results of which were encouraging. A biological effect was observed as SPC was able to antagonise substance P actions by the blocking of SP-induced vasodilation with minimal toxicity. Unfortunately, the peptide was quickly eliminated from plasma resulting in poor systemic bioavailability. This, combined with low potency of the peptide, necessitated prolonged dosing by infusion to maintain therapeutic concentrations. Such a strategy would be hard to implement clinically. Consequently, SPC could not progress to phase II trials. The low potency and rapid plasma clearance issues have since not been satisfactorily addressed.

Several other related short chain SP antagonists have been investigated in the literature. Oronz and co-workers\textsuperscript{19} designed the hexa-peptide (NY3460) sequence (e-MePhe-e-Trp-Leu-(D)-NMeIle-Leu-NH₂) and the penta-peptide (NY3532) sequence (e-MePhe-e-Trp-Phe-e-Trp-Leu-MPA) which at 50 μM both completely inhibited the growth of NCI-H69 cells in serum-free media. Recently, Sarvi et al.\textsuperscript{20} designed and synthesised a panel of eight substance P-peptide analogues ranging from a pentamer to 11-mer sequences. They compared their IC₅₀ values, relative to SPC, using H345 and H69 cell lines. All analogues were poorly cytotoxic except for the 11-mer (e-Arg-Pro-Tyr-Pro-e-Trp-Glu-e-Trp-Phe-e-Trp-Leu-MPA) which had 3 to 4-fold higher potency than SPC. The latter sequence was identical to the substance P analogue previously reported by Seck et al.,\textsuperscript{21} except for the substitution of Leu at position 10 with e-Leu, together with a non-amidated free C-terminal. The study provided crucial evidence suggesting that the modified substance P analogue can specifically target a sub-population of cells that exhibit multi-drug resistance.

Hence, we report the development of a much shorter SPC-related novel peptide with side chain modification on the indole ring of tryptophan and having the sequence o-MePhe-e-Trp-Phe-e-Trp(N-R)-Leu-NH₂. The structure is based on the straight chain C-terminally amidated pentamer sequence, 5-mer-NH₂ (1) (Fig. 1), which can be expected to be poorly active. To enhance potency, we considered regio-selectively modifying the tryptophan side chain by specifically N-terminal prenylation of the indole ring. We show that the N-terminal prenylation of the indole ring on the tryptophan residue located near the C-terminal of the penta-peptide (2) provides improved cytotoxicity against SCLC cell lines, when compared with the unmodified penta-peptide (1). This finding could be translated into in vivo efficacy by demonstrating inhibition in a xenograft model of relapsed SCLC using the DMS79 cell line.

Results and discussion

In the parent pentamer sequence 1 (Fig. 1), the tryptophan residues offer an obvious choice for side chain modification on the indole ring. Prenylated indoles are naturally found in many organisms including plants, bacteria and fungi. The prenyl moiety is usually found incorporated into the indole core as the 2,3-dimethylallyl or 1,3-dimethylallyl (tort-prenyl) substituent. A prenylated indole derivative isolated from the endophytic actinomycete streptomycetes sp. magna-405 has cytotoxic activity almost equivalent to those of demethobcin against the human lung adenocarcinoma cell line A549.\textsuperscript{22} Enzymatically driven prenylation at the indole ring of tryptophan, containing cyclic dipeptides, using recombiant prenyltransferase, produced analogues with significantly enhanced cytotoxicity against human leukemia K562 and ovarian cancer A2780 cell lines.\textsuperscript{23} Evidence is emerging to indicate that prenylation of other molecules from natural sources could offer a new spectrum of antitumor agents.\textsuperscript{24,25} Such reports, together with the presence of tryptophan residues in the pentamer sequence, provided the vision to explore N-terminal prenylation on the indole ring as a possible means to design improved SPC-related analogues for SCLC treatment. The design led to penta-peptide 2 (Fig. 1) with the incorporation of Nα-tetraprenylated tryptophan at the C-terminal end of penta-peptide 1. We synthesised the precursor Fmoc prenylated tryptophan derivatives suitable for peptide synthesis. All peptides were synthesised by standard solid phase peptide synthesis (SPPS) using Fmoc chemistry\textsuperscript{26} and peptides 1 and 2 produced were evaluated.

![Fig 1](image-url)
were also produced by liquid phase peptide synthesis (LPPS). The peptides were then screened in vitro on human SCCL cell lines (H69 and DMS79), before testing in vivo efficacy using a DMS79 xenograft model of relapsed SCCL.

**N-tert-Prenylation of Fmoc-tryptophan**

Synthesis of Fmoc-Trp(N-tert-prenyl)-OH 6 was achieved in three steps (Scheme 1) starting from Fmoc-Trp-OH 3. The carboxylic group of 3 was esterified using tetrakis(triphenyloxophosphanep) (TIP) in toluene to give 4 in 83% yield. N-tert-Prenylation of the indole ring on the tryptophan ring was achieved using Pd-mediated C-H functionalisation. The reaction involved using 40 mol% Pd(OAc)2 and 2-methyl-2-butene in the presence of Cu(OAc)2 and AgTFA as the co-catalysts in dry acetonitrile, which led to the formation of 5 in 33% yield. The succinyl group was easily hydrolysed using sodium carbonate in 50% MeCN/H2O to give 6 in 54% yield.

Other C-terminus protecting groups could be used instead of O-Su. For instance, the synthesis of 6 was carried out through the carboxylic group esterification of 3 with DCC, tert-butanol, and 4-dimethylaminopyridine (DMAP) in dry dichloromethane (DCM) to form the tert-butyl ester derivative 7 in 85% yield. N-tert-Prenylation was performed in an identical manner to above to form 8 in 68% yield. The tert-butyl group was removed using 20% TFA in DCM for 24 hours to give 6 in a 38% yield.

**Solid phase peptide synthesis (SPPS)**

SPG, peptide 1, and peptide 2 were synthesised stepwise by manual SPPS using Fmoc protecting chemistry, as described in the experimental section of the ESI, using synthesis of 2 as an example (Scheme S1). The crude peptides were purified and analysed by reverse phase HPLC (Fig. S14, S16 and S12) and characterised by mass spectrometry (Table S1). SPG gave a [M + H]2+ ion with a mass of 551.3033 Da (calculated mass: 551.3092 Da) [Fig. S15]. Peptide 1 gave a [M + H]2+ ion with a mass of 811.4290 Da (calculated mass: 811.4290 Da) [Fig. S17]. The 1H NMR spectrum of 1 [Fig. S18] gave the indole protons (N-H) of both n-tryptophan residues as singlets at 10.81 and 10.71 ppm. For peptide 2, the molecular (M + H)2+ ion at 979.4916 Da was 68 mass units higher than that for the unmodified peptide 1, confirming the presence of the tert-prenyl group. The 1H NMR spectrum of peptide 2 [Fig. S22] supported the N-tert-prenylation [N-(CH3)2CHCH2] of the indole ring of tryptophan by the appearance of an indole-NH proton, whilst retaining the unmodified indole-NH at 10.80 ppm. In addition, the appearance of a doublet of doublets at 6.06 ppm for (N-C(CH3)2CHCH2), two doublets at 5.11 and 5.10 ppm for (N-(CH3)2CHCH2), and two singlets at 1.65 and 1.60 ppm for (N-(CH3)2CHCH2) supported the presence of the tert-prenyl group. The 1H NMR assignments of peptides 1 and 2 are presented in the experimental section of the ESI. Characterisation of peptide 2 confirmed that the derivative Fmoc- Trp(N-tert-prenyl)-OH 6 is compatible with and stable to the conditions of Fmoc solid phase peptide synthesis. It is therefore a useful reagent to introduce N-prenyl groups into peptide sequences.

**Liquid phase peptide synthesis**

Liquid phase peptide synthesis is more amenable to scale-up, therefore pentapeptides were also produced by this method to establish the compatibility of Fmoc-Trp(N-tert-prenyl)-OSu 5 with this approach. The synthesis of non-prenylated peptide 1 was started at the N-terminus. Fmoc-N-Meo-Phe-OMe was treated with NHS and DCC to produce the O-succinyl activated ester, which was then coupled to the unprotected n-tryptophan under basic conditions using sodium carbonate (Na2CO3). The remaining three amino acids were incorporated, repeating the procedure of activation and coupling until the pentamer sequence 1 was formed. For the prenylated peptide 2, the fragment coupling approach was adopted. The first three amino acids of the peptide sequence (n-tripptide) were coupled as before to yield a tripeptide (Fmoc-MePhe- n-Trp-Phe-OMe) fragment. Then, a dippeptide fragment was synthesised by the reaction of 5 and benzylamine in the presence of sodium carbonate to give 9 (Scheme 2) in 47% yield which was characterised (ESI) by 1H [Fig. S23] and 13C NMR (Fig. S35) spectroscopy and mass spectrometry. The
Appendix II

Scheme 2. Synthesis of 6-Trp(2-N tert-pentyl-Leu-NH₂) 10: (a) leucinamide, Na₂CO₃, THF, (b) diethylamine, MeCN.

Fmoc was removed by treating the dippeptide with 50% diethylamine:MeCN for 1 hour to give 10 (Scheme 2). The two fragments were then coupled as above using the O-succinyl activated ester. Finally, the Fmoc group was removed as before. Both peptides 1 and 2 were purified and characterised in the same manner as that for the solid phase synthesis, giving identical analytical data.

Healey et al. documents that a broad-spectrum antagonist against SCLC, which could be used alone or in combination therapy, should ideally comprise a small non-peptide molecule based on the structure of the substance P derivative that functions similarly, but avoids the problems associated with peptide synthesis and delivery. Peptide 2 fulfills most of these characteristics as we have a short molecule (pentamer), derived from SPG, in which most of the amino acids are modified or non-natural. As it is a small molecule, its synthesis is easier to scale-up using liquid phase, particularly because the precursor N tert-pentyl tryptophan amino acid can be readily prepared in large quantities.

In vitro anti-cancer activity

Swiss Albino cell line 3T3 and two SCLC cell lines, DMS79 and H69, were selected for screening. 3T3 cells are fibroblast non-cancerous cells and were used to show the selectivity of the peptides for cancer treatment. The DMS79 cell line is a variant cell line model which is more resistant to chemotherapy as it originates from a patient with SCLC who had undergone treatment with cytotoxic, vinristine, methotrexate and radiation therapy. On the other hand, H69 represents a chemo-naive SCLC cell line model. Cell viability was assessed by quantifying the reduction of resazurin dye to fluorescent resorufin.26-31 H69 and DMS79 cells were seeded in 96-well plates at a density of 10,000 cells per well and maintained in 19% FBS/RPMI-1640 for 48 hours at 37°C in a humidified atmosphere of 5% CO₂ in the presence of various peptide concentrations. 3T3 cells were maintained under the same conditions but using 19% FBS/DMEM and a seeding density of 2,500 cells per well. The dye was then applied for 5 hours before measuring fluorescence. Cytotoxicity profiles (Fig. 2) suggest that, irrespective of the cell line used, the SPG control peptide was found to exert no inhibitory effect up to the concentration of 50 μM. In contrast, the new pentamer peptide 1 was significantly more cytotoxic on DMS79 and H69 cell lines with IC₅₀ values of 25.06 ± 2.67 and 30.74 ± 0.3 μM, respectively. Comparison of the IC₅₀ values of the pentamer with those of its N tert-pentyl modified 2 showed a further cytotoxicity enhancement of 5 to 10-fold, depending on the cell line (4.37 ± 0.44 μM on DMS79 and 2.84 ± 0.14 μM on H69), attributed to the pentyl substituent. This indicates superior in vitro efficacy compared with the recently reported 11-mer peptide 8 when benchmarked against the same cell line H69. The IC₅₀ value of 2.84 ± 0.14 μM was achieved after 48 hours of treatment with the prenylated pentamer 2 versus an IC₅₀ of 4.96 ± 0.64 μM after a longer treatment for 72 hours with the 11-mer. The peptide thus had the potential to be tested in vivo subject to its biocompatibility with plasma. Furthermore, comparing the IC₅₀ values for the DMS79 cell line with those for H69, there appears to be only a marginal difference in cytotoxicity, suggesting that the prenylated peptide may be equally effective against chemo-resistant and chemo-naive cell line models. Peptides 1, 7 and 8GG exerted no inhibitory effect on 3T3 fibroblasts, which indicates their selectivity for cancer treatment.

Dual staining by acridine orange and ethidium bromide (AO/EB) was carried out to detect tumour cell apoptosis. H69 and DMS79 cells were exposed to treatment with 6.0 μM concentration of peptides and viewed under an inverted fluorescence microscope. Untreated and SPG-treated cells largely appeared green in colour with intact nuclei. Fluorescence photomicrographs (Fig. 3 and S27) reveal that compared to SPG or peptide 1, the prenylated peptide 2 is considerably more effective at inducing apoptosis to exert its cytotoxicity. This is apparent from the larger proportion of cells being stained with ER (orange/red fluorescence) due to lost membrane integrity for both the H69 and DMS79 cell lines. Additionally, it can be seen that some cells treated with the prenylated peptide 2 exhibit sharper and more punctated yellow/green fluorescence, indicative of these cells being in the early apoptotic stage. This data corroborates the cytotoxicity data confirming the higher cytotoxicity of the prenylated peptide.

In vitro stability of peptides 1 and 2 in plasma

Plasma drug interaction affects the ability of the drug to distribute into tissues. To determine the extent of plasma binding to peptide 2, equilibrium dialysis (Cypertex Ltd) was carried out. A 5 μM solution of peptide in PBS at pH 7.5 in 0.5% DMSO was mixed with neat human plasma in a total volume of 0.15 ml. After equilibrium at 37°C, aliquots were taken from both sides of the membrane to determine the fraction of bound peptide. The mean unbound fraction of 0.728 ± 0.124 indicated that 73% of the peptide was unbound, suggesting that a reasonably high level may be available to reach tissues when administered intravenously. While in circulation, the peptide needs to be stable for a few hours to accumulate in tumours. It is commonly accepted that unmodified peptides are highly prone to protease digestion...
Appendix II

Fig. 2. Dose response curves of SPG (a, d and g), unmodified peptide (5-mer-NH₂, 1) (b, e and h) and farnesyl-NH₂ peptide 2 (c, f and i) tested in small cell lung cancer cell lines (H69 and DM579) and STS fibroblasts. Independent experiments were analysed in triplicate, n = 3. Error bars represent the standard error of the mean.

Fig. 3. Cells (top row: H69, bottom row: DM579) incubated with peptide SPG (panel 2), peptide 1 (panel 3) and peptide 2 (panel 4) at 6.0 μM for 48 h in complete medium in micro-well plates were stained with AO/EB and viewed using fluorescence microscopy. Untreated cells (left panel) were used as negative control. The scale bar on each photomicrograph is 100 μm.
in plasma, leading to rapid degradation within a few minutes. Peptides 1 and 2 were subjected to stability testing in mouse plasma. The peptides dissolved in neat plasma (0.2 ml) were incubated at 37 °C. Aliquots (20 μl) were taken at 0 and 3 hour intervals in triplicate for analysis by HPLC. Both peptides remained largely intact (Fig. 4) over a 3 hour incubation period, although a small percentage of degradation was noted for peptide 2. A plasma stability of 3 hours is considered as a sufficient time window to accumulate in tumour for efficacy studies.

in vivo anti-tumour activity

Rapid development of chemoresistance to first line treatment is a major barrier limiting the options for SCLC treatment. Evidence in the literature, published since the phase I trial for SPG, has shown that SPG can sensitize SCLC cells resistant to chemotherapy. A recent study substantiated this further by demonstrating that a novel substance-P analogue can target chemoresistant cells. DMS79 is a variant cell line derived from a patient who had received a range of chemotherapeutics and radiation, whereas H69 cells were derived from an untreated patient. DMS79 cells are therefore considered to be more likely to exhibit resistance to further therapy. Patients having variant SCLC respond unfavourably to chemotherapy and have shorter survival rates. Using DMS79 tumour-bearing nude mice as a human xenograft model of drug resistance, we tested whether peptide 2 could inhibit SCLC growth in vivo. In accordance with previously established procedures in our laboratory, ten nude female CBA mice were subcutaneously inoculated on their lower back with a suspension of 5 x 10⁶ DMS79 cells in 50% matrigel. Once tumours reached around 250 mm³, the mice were divided into two groups, half of which were treated with vehicle and half with 1.5 mg kg⁻¹ peptide 2 via peri-tumoural injection three times a week. Tumours were measured 3 times a week and harvested upon reaching 1000 mm³. Efficacy data is shown in Fig. 5, with relative % mean tumour volumes plotted against time.

The relative tumour volume (RTV) as a percentage was calculated by dividing the tumour volumes on measured days by the tumour volume on day 0, before treatment. One of the animals (dotted line, Fig. 3) in the treated group did not respond to therapy for reasons that are unclear. Excluding this single animal from the treated group, the tumour growth inhibition (TGI, Fig. 3) was calculated at day 7 as TGI (%) = (1 - T/Tc) x 100, where T indicates the mean tumour volume (mm³) of the test group and C indicates the mean tumour volume of the vehicle-treated group. At day 7, the tumour volumes of prelabeled peptide (2)-treated mice were reduced by 30% compared with the vehicle only-treated mice. This level of inhibition is significant (non-parametric Mann-Whitney U test, p < 0.0059), especially when considered in light of the small dosage given (1.5 mg kg⁻¹) as well as the chemoresistant nature of the tumour model used. In a previous study, animals bearing H69 tumours (chemo-naive) were treated peri-tumourally, daily for 1 week, with SPG peptide (45 mg kg⁻¹) and showed profound inhibition of tumour growth.

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Fig. 5. Growth of DMS79 tumours from the start of therapy at day 0 in CBA DMS79 xenografts. Once tumours reached 250 mm³, they were injected peri-tumourally with vehicle or 1.5 mg kg⁻¹ prelabeled peptide 2) 3 times a week. The control and treated groups each contained 5 mice. Data for the control group is shown as the mean ± SE (shaded error bars in grey). Data for the treated group is shown as individual animal data.
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Given that the cell line used in our study originated from a patient who had received a number of chemotherapeutics as well as radiation therapy, and the doses of peptide used were 30 times lower than those of the SPG antagonist, the observed effect is extremely promising. The % TCG value declined consistently throughout the treated period, with the initial reduction in tumour growth observed from as early as day 2 of treatment. In contrast, the control group showed consistent rapid increase in mean tumour volumes. A more recent study9 used an alternative substance P analogue comprising an 11-mer sequence and tested its in vivo efficacy using H446 xenografts, challenged with subcutaneous injections of the peptide at a dose of 25 mg kg⁻¹. The 11-mer peptide was able to induce a significant reduction in tumour growth, comparable with eroposide treatment. However, the 11-mer peptide was used at 17 times higher dose than peptide 2 in this study. Unfortunately, the limited aqueous solubility of our peptide 2 prevented further dose increase to demonstrate its full potential. Our attempts to dissolve the peptide at higher concentration failed due to significant precipitation making it unsuitable for reliable administration. This may be overcome by a formulation design using a different vehicle. Liposome encapsulation may offer a better approach to evaluate its in vivo performance as such a composition may overcome solubility issues, by embedding in the lipid bilayer, as well as assisting tumour accumulation. In this context, a liposome delivery approach for the SPG peptide showed an increased cellular association with H69 cells,29 which will be evaluated with the prenylated peptide 2. Other approaches such as the use of cyclodextrin to aid solubility may offer further scope. Indeed, the peptide has a small size and multitude of hydrophilic side chains to potentially complex with cyclodextrins.40,41

The prenylated peptide is based on the SPG sequence for which there is no single target receptor known and being a broad-spectrum antagonist there is no single mechanism to account for its mode of action. Based on its precursor sequences (SPG), the prenyl peptide (2) likely also functions as an antagonist of several neuropeptides which are known to promote mitogenesis in cancer cells. The ligand receptor interactions at a molecular level for SPG are however unknown. It has been occasionally speculated42 that the substance P broad-spectrum antagonists may recognise a common domain on Gα mobilising neuropeptide receptors. However, there is no structural elucidation of any such discrete structure. Given that there may be several prospective receptors involved and its mode of action could also involve binding to other proteins to regulate such receptors, it is not feasible at this stage to minimise, on the basis of any molecular modelling, as to why the prenylated indole gives better activity. However, based on the in vitro cytotoxicity assays, showing the 5-mer sequence (1) to be relatively inactive, it could be hypothesised that prenylation, rendering the indole ring more lipophilic, could enhance binding with at least some neuropeptide receptors through hydrophobic interactions involving the Trp (4th residue) side chain. Additionally, in the case of the N-prenyl peptidase (2), the indole NH group on this residue would lose its ability as a hydrogen bond donor, which, once bound, may affect the structure and function of the receptors involved. The better activity in relation to the SPG analogue could also be due to the smaller size as well as the increased lipophilicity of the prenyl peptide (2) to help case binding with the receptors. It cannot be ruled out whether the N-prenyl moiety on the indole could also function at a non-receptor level by binding to other proteins which may participate in the neuropeptide-promoted mitogenesis.

Conclusion

Methods were developed to establish the synthesis of N-prenyl analogues of tryptophan on the indole ring. The resulting N-prenyl-protected tryptophan amino acid analogues were compatible with solid phase and liquid phase peptide syntheses. The clinical need for new therapeutics against SCCL is compelling due to poor prognosis compared to many other cancers. The broad-spectrum antagonism concept offers hope for a multi-target approach by exploiting the neuroendocrine nature of this cancer, in which tumour cells produce neuropeptides that act as autocrine and paracrine growth factors. We transected the well-known SPG peptide to a shorter pentamer sequence amenable on the C-terminal and enhanced its cytotoxicity by more than an order of magnitude compared with SPG, through N-prenylation of the tryptophan residue. The prenylated peptide (2) was found to resist degradation by plasma for at least 3 hours and had in vivo antitumour activity at relatively low doses (1.5 mg kg⁻¹) against DMS79 xenograft growth. Here, peptide 2 is reported, as the smallest, simplest, most active and stable SPG analogue, with broad-spectrum antagonist activity. As in vitro and in vivo activity was noted on DMS79 cells, it is possible that the peptide may have the potential to target chemo-resistant cells comparable to a recently published sequence19 also derived from SPG. The in vitro delivery of peptide 2 will need to be improved due to its lower aqueous solubility than SPG and encapsulation in liposomes in a similar fashion to post-insertion of D1 by14 may comprise a suitable vehicle to overcome this issue. Finally, prenylated indoles are naturally found in many organisms, therefore the feasibility of having prenylated tryptophan analogues suitable for peptide synthesis may open up opportunities to exploit this modification to improve the biological activity of other synthetic peptides.

Ethics statement

All procedures involving animals were performed in accordance with the UK Home Office Animal (Scientific Procedures) Act 1986, and approved by the local University of Manchester Ethical Review Committee.
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Acknowledgements

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Notes and references


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Pentapeptides for the treatment of small cell lung cancer: Optimisation by N\textsuperscript{\textit{nd}}-alkyl modification of the tryptophan side chain

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ABSTRACT

The pentapeptide, tert-Val(DTP)-Ile(DTP)-Val(DTP)-Ile(DTP)-NH\textsubscript{2}, has recently been developed by our group to exhibit properties of substance P (SP) antagonist against small cell lung cancer (SCLC). In this study, we undertook a systematic structure activity investigations to optimise the lead compound to improve its in vitro anti-tumour activity and bioavailability. A series of DTP derivatives were synthesised, with a range of aliphatic N-alkyl chains (methyl to propyl) on the indole ring 4-N. These were incorporated into the pentapeptide sequence by substitution of the N4-tert-propylated DTP residue with N\textsuperscript{\textit{nd}}-alkyl DTP derivatives. These pentapeptides were significantly more potent than tert-Val(DTP)-Ile(DTP) with the N\textsubscript{4}-butyl modification generating the most cytotoxic peptide. Compared to tert-Val(DTP)-Ile(DTP), a single butyl modification on the 4-N DTP residue (Butyl-DTP-NH\textsubscript{2}) showed a 1.5-fold enhancement in cytotoxicity in either the chemo-sensitive H69 or the DMS575 (originating from a patient treated with chemotherapy and radiation therapy) SCLC cell lines. In addition, the di-alkylated sequence on the 2nd and 4th DTP residues (Butyl-N4-Butyl-NH\textsubscript{2}) gave 45 times higher cytotoxicity against both the H69 cell line and a 2-fold increase against the DMS575 cell line, compared to tert-Val(DTP)-Ile(DTP). The latter position for butyl modification was the 4th DTP residue, whereas the former position gave lower cytotoxicity on both cell lines. Radiolabelled peptide sequence, when exposed to normal mouse plasma for 24 h at 37°C, was found to be unstable with a half-life of 17 min for both the 4th and 5th DTP residues. The degradation pathway in plasma is via deamination of the C-terminus, confirmed by mass spectrometry and HPLC analysis. The butyl modification considerably increased the resistance to metabolism when tested using 39 liver function from mice. The optimum analogue responsive against the DMS575 cell line was the Butyl-DTP-NH\textsubscript{2} pentapeptide, which revealed a concentration dependent increase in apoptosis; the level of late apoptotic cells rose from 38% at 2 μM to ~9% at 5 μM, as determined by flow cytometry, compared to the unmodified peptide that showed no such effect. Concluding, the tumour substitutions offered the best perspective for high cytotoxicity, induction of apoptosis and metabolic stability thereby comprising an improved broad spectrum SP antagonist candidate for treatment of SCLC.

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1. Introduction

Affecting mainly smokers, small cell lung cancer (SCLC) accounts for almost one-quarter of all cancer deaths worldwide [1-4]. First-line treatment, entailing a combination of chemotherapy (etoposide and cisplatin or carboplatin) and radiotherapy, is initially efficacious but then short-lived [3,5]. The vast majority of patients relapse and develop resistance [3,6]. Moreover, SCLC manifests early widespread development of metastases, often before the patient has been diagnosed with lung cancer [7]. Hence, it remains one of the most aggressive forms of cancer and any new advances in developing chemotherapeutics have made little impact on patient outcome [1]. Existing cytotoxic agents that target a highly specific receptor suffer from rendering the cells chemoresistant. To overcome this obstacle, scientific approaches based on inhibiting mdr targets are needed to alleviate drug resistance and achieve better efficacy. SCID is a pulmonary neuroendocrine carcinoid tumour. These cells produce a wide variety of neuroendocrine growth factors and their cognate receptor. Several neuroendocrine growth factors,
including gastrin releasing peptide (GRP), neuropeptide Y, gastrin, cholecystokinin, neurotensin, vasoactive intestinal peptide and bradykinin, have been implicated in self-promoting tumour growth by acting through autocrine growth loops [8–10]. For example, they cause intracellular GTP
c

obilisation that activates further signal transduction pathways [11–15]. In addition, the expression of these neuropeptide receptor increases as cells become resistant to first line chemotherapy [14]. Therefore, it is conceivable to interfere with the growth of SCCL using drugs which may block the action of these neuropeptide antagonists, refered to as substance P (SP) analogues, that interrupt multiple neuropeptide signaling pathways to inhibit the proliferation of SCCL have been explored for their potential as broad spectrum anticancer agents. These SP analogues are well recognized competitive inhibitors of the mitogenic effects of several different neuropeptides. Among these analogues are: [D-Arg5,D-Phe8]-Leu-enkephalin (SPD) [15–17], [D-Arg5, D-Phe8]-Me-O-SP (5–11) [17–19], [D-Arg5,D-Phe8]-Leu-enkephalin (DFAE) [20], N,N-Diethyl-N-[D-Arg5,D-Phe8]-Leu-enkephalin (ENLK) [21] and [D-Arg5,D-Phe8]-D-Ala7- Leu-enkephalin (NLK) [22]. The exact mode of action of the SPD analogues remains unclear and may involve several G protein coupled receptors (GPCRs), for example, GRP receptor, vasoactive intestinal peptide and tachykinin NK1 receptor [15,17,23,24].

Cancer stem-like cells (CSCs) belong to a small subpopulation of persistent cancer cells with acquired resistance to standard chemotherapy and research [24] points to CSCs playing an important role in the pathogenesis of SCCL. CD133 is a known biomarker of CSC in several cancers. Savic and co-workers [22] found that CD133 positive cells had a higher level of GTP and vasoactive intestinal peptide receptors and yet these cells also exhibit increased sensitivity to an SP analogue which is related to sequence in SPD. SPG is the only neuropeptide to have been tested on human subjects in a phase 1 clinical trial [25]. These findings suggest a potential for dual efficacy by not only interfering tumour growth per se, but also overcoming drug resistance. While many synthetic analogues have been designed and tested on a number of SCCL cell lines and tumour models [15–22], the in vitro potency and hence in vivo tumour response at low doses remains far from satisfactory.

Recently, we have developed a novel peptide derived from SCCL but with a shorter amino acid sequence, 5-mer-NH2 (1) [D-Phe8-D-Arg5-Phe-D-Arg5-Leu-NH2], with IC50 values in the range of 2.3–31 μM against H69 (chemo-resistant SCCL line) and DMS79 (SCCL cell line originating from patient treated with chemotherapeutics and radiation therapy), superior to SPD [26]. Cytotoxicity was greatly enhanced by chemical tert-tryprenyl modification of the indole nitrogen (N10) of the 4th-4-tryprenyl (D-Trypt) residue to comprise tert-tryprenyl-4-Arg(5) (2) [D-Me-O-D-Phe8-D-Arg5-Phe-D-Arg5-Leu-NH2] [26]. The latter compound exhibited IC50 values of 2.8±0.4 μM and 4.37±0.44 μM on H69 and DMS79 cells, respectively [26]. Under the same conditions, SPD did not show cytotoxicity up to the maximum tested concentration of 90 μM on either cell line [26]. Using DMS79 spheroids and applying a low dose of 1.5 mg/mL, a 10% reduction in tumour volume was evident [26]. The objective of this research is to further enhance the cytotoxicity of these peptide analogues. Hence, inspired by the remarkable effect of the introduction of the tert-trypenyl moiety to increase the potency of the peptide sequence against SCCL, we aimed to optimize the lead structure by considering increasing lengths of aliphatic alkyl modifications on the D-Trypt residue. Here, we have generated a further eight pentapeptides and explored the possibility of having the 2nd D-Trypt residue modified. The most cytotoxic candidates were tested for their stability in plasma and 25% liver fraction from mouse before assessing their ability to induce apoptosis.

2. Results and discussion

2.1. Synthetic of N10-alkylated 4-trypenyl derivatives

The synthesis of N10-alkylated D-Trypt derivatives are illustrated in Scheme 1. The tert-butylxyloxyctonine D-Trypt (Boc-D-Trypt-OH) was treated with two equivalents of potassium tert-butoxide to deprotect N10 and the carboxylic acid, followed by reaction with allyl (methyl, ethyl, propyl, butyl and pentyl) iodide. This resulted in alkylation of N10 whilst also forming the allyl ester (Boc-D- Trypt(allyl)-OH) (4–7). Subsequently, the ester was hydrolysed forming the alkylated building block required for peptides synthesis (Boc-D-Trypt(allyl-OH)) [8–12]. The structures were confirmed by 1H and 13C NMR spectroscopy and mass spectrometry (MS) as reported in the experimental, with NMR spectra provided in the Supplementary data (SD) (Fig. S1–S14).

The 1H NMR (400 MHz, CDCl3) spectra for the modified derivatives showed the disappearance of the N10-8 (observed at 8.09 in the un-modified Boc-D-Trypt-OH) and the appearance of the peak corresponding to the added alkyl groups, for example, the spectrum for 3 showed the appearance for two peaks corresponding to the two added methyl groups at 3.75 and 3.69 ppm, and the disappearance of the N10-H peak (Fig. S3A). The spectrum for the tert-butyl (4) showed only a single peak for the methyl group on N10 at 3.74 ppm (Fig. S7A).

2.2. Peptides synthesis

The synthesis of peptides (13–20) (Fig. 1) was completed using liquid phase peptide synthesis (LPPS) as previously described [26]. The procedure entailed activating the free carboxyl group to produce N-hydroxy succinimide (NHS) amino acid ester or (short chain NHS peptide ester after amino acids coupling), followed by coupling with free amino acid (Scheme 2). Peptides were purified by reversed phase (RP)-HPLC (≥95% purity) and structures (13–20) were verified by MS (data in experimental section and RP-HPLC chromatograms in Fig. S12–S19).

2.3. Cell viability assays

Cell viability was evaluated by treating H69 and DMS79 cells with peptides 13–20 in the 0.1–30 μM range for 48 h, with subsequent use of the resazurin dye cell viability assay. IC50 values (Table 1) for all the peptides were derived from dose response curves (Fig. 2) and presents the IC50 values obtained for 1 and 2 previously [26]. In the first series of peptides (13–17), the N10-alkylated D-Trypt is located near the C-terminus of the peptide sequence (4th residue), analogous to the previous lead sequence (2) [26] that was modified with tert-trypenyl at the same position. Benchmarcking the observed IC50 values (Table 1) against 2, all of the N10-alkyl modified peptides in this series (13–17) showed higher cytotoxicity as both cell lines, except for 13 (N10-methyl-D-Trypt) on H69, which was 1.4 times less active. There is a general trend for increased cytotoxicity as the alkyl chain length increases from C3 to C4. The best analogue comprises N10-buty-D-Trypt at the 4th residue (16). Overall, 16 was 5 fold more cytotoxic, in vitro, on both cell lines than 2.

To explore whether 1-D-Trypt on the 2nd residue of the sequence mimics the peptide more dynamic, 18 was synthesised and evaluated. This positional change of N10-buty-D-Trypt in the peptide sequence reduced the potency by approximately 3–4 fold, in comparison to 16, on both cell lines. Its cytotoxicity was comparable to the methylated analogue (13). However, 16 and 18 are considered potent in comparison to 1 that has un-modified D-Trypt residues (Table 1). In view of this, a further peptide was designed to take
Appendix II

![Chemical structures and reaction schemes]

Table 1

<table>
<thead>
<tr>
<th>Peptide</th>
<th>H9/H9 Cells</th>
<th>DMN Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 ± SE (nM)</td>
<td>IC50 ± SE (nM)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>36.74 ± 0.30</td>
<td>23.06 ± 2.07</td>
</tr>
<tr>
<td>2</td>
<td>5.84 ± 0.14</td>
<td>4.27 ± 0.44</td>
</tr>
<tr>
<td>3</td>
<td>3.03 ± 0.22</td>
<td>4.03 ± 0.31</td>
</tr>
<tr>
<td>4</td>
<td>1.48 ± 0.03</td>
<td>1.46 ± 0.03</td>
</tr>
<tr>
<td>5</td>
<td>1.37 ± 0.10</td>
<td>1.31 ± 0.10</td>
</tr>
<tr>
<td>6</td>
<td>1.40 ± 0.22</td>
<td>1.43 ± 0.18</td>
</tr>
<tr>
<td>7</td>
<td>1.30 ± 0.10</td>
<td>2.17 ± 0.06</td>
</tr>
<tr>
<td>8</td>
<td>4.03 ± 0.17</td>
<td>2.96 ± 0.15</td>
</tr>
<tr>
<td>10</td>
<td>0.63 ± 0.05</td>
<td>2.31 ± 0.10</td>
</tr>
<tr>
<td>20</td>
<td>&gt;30</td>
<td>&gt;30</td>
</tr>
</tbody>
</table>

A further analogue of the most potent peptide (16) against DM579 was produced to study the influence of the C-terminal amide group. Peptide 20 was thus synthesized as a free carboxylic acid and screened. The de-amination of the C-terminus completely inactivated the peptide against both cell lines, with no growth inhibition at 30 nM. This indicates that the C-terminal amide is crucial for cytotoxicity.

Broad spectrum antagonists may have multiple targets belonging to the G-protein linked receptors, many of which are membrane bound with unknown crystal structures. In the absence of specific target and structural information, it remains difficult to rationalize the exact role played by the N-terminal and the C-terminal amide groups on the basis of molecular modeling. However, since the cytotoxicity peaked at the same chain length (butyl) for both cell lines, it could be argued that there is some common binding site requiring strong hydrophobic interaction with an alkyl chain and such site has some constraints to optimally

![Chemical reaction schemes]
Appendix II

accommodate butyl group. This may also explain the loss of activity upon de-amidation, as the carboxylic group in its ionised form would significantly reduce the hydrophobicity gained from the alkyl chain that is critical to render the peptide cytotoxic. The hydrophilic-hydrophobic balance is particularly effective as the 4th residue is close to the peptide’s C-terminus.

2.4. The stability of peptides 216, 19 and 20 in plasma and 50 liver fraction from mouse

When optimizing for functional activity there is also a need to assess whether the desired modification will negatively affect the stability of the peptide. Characterization of the metabolism of peptides is necessary to improve their stability in vivo. The finding that a carboxylic acid group, instead of the usual amide, at the C-terminus mitigates the cytotoxicity of 16 (results of 216 - Table 1) may compromise its use in vivo as protein de-amidation reactions are widespread in plasma. Peptides 16 and 19, being the most cytotoxic peptides, were exposed to the conditions of in vitro metabolite in plasma. Peptide 20 lacking the amide group was used as a control. Peptides 16, 19 and 20 were incubated in neat mouse plasma at 37°C for 48 h. Peptide 2, previously tested over a shorter incubation time [27], was included for benchmarking purpose. Relevant sections of typical chromatograms obtained are shown in Fig. 2.

The chromatograms for 216 and 19 show a gradual loss of the parent peptide with the concomitant increase in intensity of closely eluting peak(s) with a slightly higher retention time (tR); suggesting time dependent degradation in plasma. Quantitative assessment (Fig. 3) revealed that the byproduct sequences (16 and 19) were far more stable with >80% integrity compared to the non-processed (216) form, which was almost 40% after 24 h exposure. The de-butylated peptide (19) was the most stable with 81% integrity after 48 h exposure to neat plasma. Peptide 20, similar to 16 but with a carboxylic acid at the C-terminus, showed no sign of degradation. The percentages of the parent peptides remaining after each time point in plasma studies are presented in SD Table S1.

From the closely matching retention times for the degradation product of the amidated peptide 16 (tR 25.23 min after 48 h) and that of the carboxylic acid peptide 20 (tR 23.27 min after 48 h), it seems certain that the degradation of 16 must arise from a de-amidation reaction in plasma. To substantiate this further, both peptides (16 and 20) were mixed and processed using identical sampling procedures. The amount of 20 in the mixture was kept at 2.5 times lower than 16 to simulate higher levels of the amidated product as noted for the 48 h plasma treated sample (Fig. 2, bottom RP-HILIC trace section for 16) and to help assign the two peaks following elution. The mixture of the two peptides was exposed to neat plasma as above and the samples analysed. Fig. 4 clearly showed that the de-amidated form had a peak of lower intensity, which eluted later than its amidine form, the relative percentage of each compound and their specific is also presented beneath the chromatographic sections. The profiles and degradation pattern appears to similar those obtained for the degradation of 16 (Fig. 2) having almost identical tR values. The peak area assigned to the de-amidated peptide 20 increases concurrently with a decline of the amidine peptide 16. The process of degradation in plasma is thus likely to be due to de-amidation of the C-terminus residue.

Samples at 0 and 48 h incubation with plasma (Fig. 4) were analysed by MS. The MS spectra (presented in SD) also supports the de-amidation of 16 to 20. The most abundant ion observed for the de-amidated sample at m/z 808.5 (Fig. S30) compared to m/z 867.5 (Fig. S30) for the amidated compound for the 0 h sample. Results were similar for the plasma stability study for 2 showing a one unit higher mass after incubation in plasma. The predominant ion at m/z 808.5 (Fig. S31) was present at 48 h for the de-amidated compound and at 0 h for the parent amide peptide (2) at m/z 867.5 (Fig. S31). Peptide 20, devoid of C-terminus amide, showed the same predominant ion at m/z 867.5 (Fig. S32) indicating complete stability in plasma, in agreement with the RP-HILIC data (Fig. 2). For 16 and 19, the MS results showed a predominant ion for the parent amidated form at 0 and 48 h (Fig. S33 and S34). This is consistent with the observed RP-HILIC profiles (Fig. 2) also indicating that the major component remaining is the native butylated and amidated sequences. The Nα-de-butyryl modifications have thus significantly reduced the susceptibility of these analogues to plasma, to a point that there is little peptide degradation up to 48 h.

Peptides 16 and 19 were further subjected to the 50 liver fraction from mouse in the presence of cofactors [27] for a period of 3 h. This would give further insight into the stability of the peptides towards Phase I and Phase II metabolizing enzymes. Peptides 16 and 19 were shown to be completely stable in 50 liver fraction (Fig. S35 and S36).

Our results are consistent with another related SP analogue known as SPUT, which most likely targets the same receptors as SP-28. One of the two major metabolites for SPUT (Arg-Pro-Lys-Pro-DIle-Val-Glu-DPhe-Cys-DAPA) was isolated and confirmed by MS as the C-terminus de-amidated product (DIle-Val-Glu-DPhe-Cys-DAPA-Leu-NH2) as a result of serine protease action. In vitro biological activity of this metabolite was poor as the action of neuropeptides, bombesin, vasopressin or bradykinin could not be antagonised with it. This is in contrast to the antagonist effect observed with the amidated parent peptide when using the same neuropeptide. It was implied that receptor for these growth factors could be more selective in binding to the C-terminus and thus result in this antagonistic effect. Our results support this proposal as modifications near the C-terminus (DIle-Val) at 4th position, were identified as the most effective sites to maximize the cytotoxicity and retain on plasma and 50 liver fraction degradation. Therefore the hypothesis made by Jones et al. [28] that “development of more potent broad-spectrum antagonists may be possible by slight modifications of the C-terminal” has now been substantiated with our analogues in this study.

2.5. Assessment of apoptosis

2.5.1. Actinide orange (AO)/hemitrichrome double staining

The most cytotoxic peptides, singly (16) and de-butylated (24) peptides, were selected for testing their ability to induce apoptosis in HEK and DMS/29 cells lines. Photomicrographs of cells stained with actinide orange (AO) and hemitrichrome (3B) are presented in Fig. 5. Untreated cells (Fig. A and B) showed predominately green fluorescence due to intact plasma membrane and Alexa Fluor 647 labelled actinide orange nuclei, with almost red-orange fluorescence attributed to loss of plasma membrane integrity is seen [29]. Hence, EB gains entry into cells to interact with the DNA, highlighting the late apoptotic and necrotic cells [24,30]. In the latter case (afp+gM) cell detachment was also observed when compared to the controls, suggesting apoptosis [31]. A few bright green regions were still observed on cells treated with 6 µM, suggesting chronic condensation of cells underlying apoptotic [30].

2.5.2. Annexin V conjugate and flow cytometric analysis

The novel potent Nα-de-butyrylated peptide, 16, and its unmodified version 1 [30] were incubated for 24 h with DMS/29 cells and stained using Annexin V Fluorescein 555 (Avio) conjugate and SYTOX Blue (SyB) dead cell stain [32,33]. The former stains binds to phosphatidyl serine (PS) residues that become exposed at the outer
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![Diagram with peaks and time points](image)

Fig. 2. Sections of HPLC chromatograms obtained from plasma stability study for 2, 16, 19, and 20. Chromatograms are from 0, 24, and 48 h post-injection of peptides at 37°C with retention times (t_r) of peaks observed presented beneath the chromatograms. The full chromatograms are presented in 30 (Fig. S29-S28).

Quantitative assessment of apoptosis was evaluated at low (2 µM) and high (8 µM) peptide concentrations using flow cytometry. A bar-chart (Fig. 6) presents the levels of cells classified into four stages: live cells (AnnV-/Syb−), early apoptotic cells (AnnV+/Syb−), late apoptotic cells (AnnV+/Syb+) and necrotic cells (AnnV−/Syb+). Dot plots are presented in the SD (Fig. S37). Apart from a slight increase in live cells, treatment with 1 had similar levels of cells in each of the four stages as the untreated sample, irrespective of the concentrations used. This is an expected observation as 1 has a high IC_{50} value (21 µM) [28] on this cell line and will be non-cytotoxic at these concentrations. In contrast, the ringyl butylated peptide (16) caused highly significant (P < 0.0005) apoptosis in a concentration dependent manner. Even at a low concentration of peptide (2 µM), a small increase (P < 0.05) of early apoptotic cells was observed relative to the control sample. Raising the concentration of 16 to 8 µM caused the level of late apoptotic cells to increase massively up to 65.5% (Fig. S37 E – top right quadrant) and concurrently the level of viable cells (Fig. S37 E – bottom left quadrant) dropped to 10%. These results show that the optimised peptide 16 was highly effective to induce cell death in what would
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Figure 3. Bar chart presentation for the relative percentage (±SEM) of 2, 8a, 2b, 20 to degradation product(s) in plasma studies at 0, 3, 24 and 48 h post-inoculation; n = 3.

Figure 4. Section of HPLC chromatograms for the plasma stability study performed on the mixture containing 30% of 2b, a lower amount of 3b, over time (0–48 h). The percentage of each peak is presented beneath each chromatogram. The full chromatograms are presented in SI (Fig. S20).

Figure 5. IMCD cells (top) and IMCD70 cells (bottom), untreated (A and D) and incubated with 0.1 μM of 3b (B and E) and 10 μM (C and F) for 48 h in complete media in 6-well plates. The cells were stained with 5 μM AMOCM reagent and viewed under an inverted fluorescence microscope. The scale bar on each photomicrograph is 100 μm.

otherwise be a very difficult cell line (IMCD70) to treat using conventional chemotherapy.

3. Conclusion

Novel pentapeptides based on the short 5′-NG sequence were synthesised by incorporating D-Trp derivatives modified with Nα-butyryl chains. Screening against SCC cell lines revealed that the Nα-butyryl substituent provided the most cytotoxic peptides with improved resistance to cleavage by plasma and metabolism in the NIH HeLa fraction. A single Nα-butyryl modification on the 4th D-Trp residue enhanced the cytotoxicity by a ~3 fold with IMCD and IMCD70 cells, compared to our recently discovered sequence (tert-PrenylNH2) [28] where tert-Prenyl was incorporated on the
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A solution of n-BuOK (0.74 g, 6.58 mmol) in dry toluene (5 mL) was added dropwise to a solution of 2-iodo-2H-tetrazole (10 g, 52.80 mmol) in dry N,N-dimethylformamide (10 mL) under N2 atmosphere at 0 °C. The mixture was stirred for 20 min at room temperature (RT), isododecanite (490 mL, 0.58 mmol) was then added to the above mixture under N2 atmosphere at 0 °C. The mixture was stirred at RT for 8 h. The reaction completion was checked by TLC (ethyl acetate/hexane 1:1). Concentrated aqueous solution (30% w/v) (200 mL) was then added to the mixture and the crude product was purified by flash column chromatography (ethyl acetate/hexane 1:4), to give 6.75 g (85.7%) of a yellow oil. 

4. Experimental

4.1. Materials and instrumentation for the synthesis of N\(^\text{3}\)\textsuperscript{-}N\(^\text{o}\)\textsuperscript{1} dialkylated \(\alpha\)-tryptophan derivatives

Chemicals and solvents were obtained from Nacalai Tesque, Sigma-Aldrich and Fisher Scientific. Silica gel, ZEOPREP 60 (40-63 \(\mu\)m), was obtained from Apollo Scientific. Thin layer chromatography (TLC) plates TLC Silica gel 60 F\(^{254}\) was obtained from Merck Millipore. Reaction progress was monitored by TLC and the spots on TLC plates were visualized using UV Minelamp light (365/935) (365 nm) and 75 MHz (\(75^1\)H) spectrometer and chemical shifts (\(\delta\)) are quoted in ppm per million and referenced to solvent residual peak. NMR spectra were generated using TopSpin 2.1 software. Electrospray Ionization MS (ESIMS) Waters SQ-2 single quadrupole mass spectrometer attached to Acquity UPLC and Atmospheric Pressure Chemical Ionization MS (APCI-MS) (Agilent Technologies 6120 Quadrupole LC/MSD) were performed at the School of Chemistry, the University of Manchester and molecular ion peaks are reported as mass/charge (m/z) ratio. Melting points (mp) were measured using a Smart Scientific melting point apparatus SMP10.

4.1.1. Boc-2-Tyr\((\text{N}-\text{methyl})\)-D-methyl (3)

A solution of n-BuOK (0.74 g, 6.58 mmol) in dry toluene (5 mL) was added dropwise to a solution of Boc-2-Tyr\(-\text{CH}_2\text{-OH} \) (10 g, 52.80 mmol) in dry N,N-dimethylformamide (10 mL) under N\(_2\) atmosphere at 0 °C. The mixture was stirred for 20 min at room temperature (RT). Isododecanite (490 mL, 0.58 mmol) was then added to the above mixture under N\(_2\) atmosphere at 0 °C. The mixture was stirred at RT for 8 h. The reaction completion was checked by TLC (ethyl acetate/hexane 1:1). Concentrated aqueous solution (30% w/v) (200 mL) was then added to the mixture and the crude product was purified by flash column chromatography (ethyl acetate/hexane 1:4), to give 6.75 g (85.7%) of a yellow oil. 

4.1.2. Boc-2-Tyr\((\text{N}-\text{ethyl})\)-D-ethyl (4)

The method of synthesis was similar to that described for 3, except that isododecanite (490 mL, 0.58 mmol) was used. A yellow oil (6.67 g, 56.5%) of 4 was isolated after flash chromatography. 

4.1.3. Boc-2-Tyr\((\text{N}-\text{propyl})\)-D-propyl (5)

The method of synthesis was similar to that described for 3, except that 1-iodopropane (642 mL, 6.58 mmol) was used. A yellow
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oil (0.75 g, 5.86% of 9) was isolated after flash chromatography. 1H NMR (400 MHz, CDCl3) δ 7.56 (d, 1 H, J 8.0 Hz, Ar-H), 7.30 (d, 1 H, J 8.0 Hz, Ar-H), 7.20 (dd, t, 1 H, J 7.6 Hz, 7.4 Hz, Ar-H), 7.00 (d, 1 H, J 7.6 Hz, Ar-H), 6.00 (s, 1 H, Ind-2-H), 5.06 (br, s, 1 H, J 10.0 Hz, NHCCH3), 4.62 (dd, 1 H, J 13.5, 5.3 Hz, CH3) 4.00 – 3.90 (m, 9 H, NCH2CH2CH2CH2ON in ind and ester), 3.32 – 3.21 (m, 2 H, CH2), 1.80 (pentet, 2 H, J 2.8 Hz, NCH2CH2CH2CH2ON in ind). 13C NMR (75 MHz, CDCl3) assignments made using DEPT-135 δ 172.5 (C-12), 155.3 (C-15), 136.4 (C-9), 128.5 (C-10), 121.2 (C-11), 119.1 (2 x C-6a), 109.3 (CH-Ind), 108.8 (C-14a, C-14c), 107.4 (C-14b). 2C NMR (125 MHz, CDCl3) assignments made using DEPT-135 δ: 176.8 (C-12), 155.8 (C-15), 137.1 (C-6a), 132.8 (C-9), 131.9 (C-10), 127.3 (CH-Ind), 121.2 (C-11), 119.4 (C-14a, C-14c), 119.2 (CH-Ind), 109.3 (C-14b). 104.9 (C-14c), 108.3 (C-14a), 104.9 (C-14b). 32.8 (CH3-NHCCH3). 20.4 (CH3, CH2, C15, C17), 27.6 (CH2, C10, MS (ESI) m/z [M+Na]+ 4141.04).

1.4. Boc-D-Tyr[-boc]-boc-D-tyr (6)

The method of synthesis was similar to that described for 3, except that 1-tido-2-benzene (294.94 mmol) was used. A yellow oil (1.03 g, 76.9%) of 6 was isolated after flash chromatography. 1H NMR (400 MHz, CDCl3) δ 7.25 – 7.55 (4 H, 1H, J 7.7 Hz, Ar-H), 7.30 (d, 1 H, J 8.4 Hz, Ar-H), 7.20 (dd, t, 1 H, J 7.7 Hz, Ar-H), 6.90 (s, 1 H, Ind-2-H), 5.07 (br, s, 1 H, J 10.4 Hz, NHCCH3), 4.61 (dd, 1 H, J 13.6, 5.6 Hz, CH3) 4.10 – 4.03 (m, 4H, NCH2CH2CH2ON in ind and ester). 13C NMR (75 MHz, CDCl3) assignments made using DEPT-135 δ 172.5 (C-12), 155.3 (C-15), 136.4 (C-9), 128.5 (C-10), 121.2 (C-11), 119.1 (2 x C-6a), 109.3 (CH-Ind), 108.8 (C-14a, C-14c), 107.4 (C-14b). 2C NMR (125 MHz, CDCl3) assignments made using DEPT-135 δ: 176.8 (C-12), 155.8 (C-15), 137.1 (C-6a), 132.8 (C-9), 131.9 (C-10), 127.3 (CH-Ind), 121.2 (C-11), 119.4 (C-14a, C-14c), 119.2 (CH-Ind), 109.3 (C-14b). 104.9 (C-14c), 108.3 (C-14a), 104.9 (C-14b). 32.8 (CH3-NHCCH3). 20.4 (CH3, CH2, C15, C17), 27.6 (CH2, C10, MS (ESI) m/z [M+Na]+ 4141.04).

1.5. Boc-D-Tyr[-boc]-boc-pyroxylin (7)

The method of synthesis was similar to that described for 3, except that 1-tido-proline (38.66 g, 0.25 mmol) was used. A yellow oil (0.75 g, 36.3% of 7) was isolated after flash chromatography. 1H NMR (400 MHz, CDCl3) δ 7.54 (d, 1 H, J 8.0 Hz, Ar-H), 7.30 (d, 1 H, J 8.0 Hz, Ar-H), 7.20 (dd, t, 1 H, J 7.6 Hz, 7.4 Hz, Ar-H), 7.00 (d, 1 H, J 7.6 Hz, Ar-H), 6.00 (s, 1 H, Ind-2-H), 5.06 (br, s, 1 H, J 10.0 Hz, NHCCH3), 4.62 (dd, 1 H, J 13.5, 5.3 Hz, CH3) 4.00 – 3.90 (m, 9 H, NCH2CH2CH2CH2ON in ind and ester), 3.32 – 3.21 (m, 2 H, CH2), 1.80 (pentet, 2 H, J 2.8 Hz, NCH2CH2CH2CH2ON in ind). 13C NMR (75 MHz, CDCl3) assignments made using DEPT-135 δ 172.5 (C-12), 155.3 (C-15), 136.4 (C-9), 128.5 (C-10), 121.2 (C-11), 119.1 (2 x C-6a), 109.3 (CH-Ind), 108.8 (C-14a, C-14c), 107.4 (C-14b). 2C NMR (125 MHz, CDCl3) assignments made using DEPT-135 δ: 176.8 (C-12), 155.8 (C-15), 137.1 (C-6a), 132.8 (C-9), 131.9 (C-10), 127.3 (CH-Ind), 121.2 (C-11), 119.4 (C-14a, C-14c), 119.2 (CH-Ind), 109.3 (C-14b). 104.9 (C-14c), 108.3 (C-14a), 104.9 (C-14b). 32.8 (CH3-NHCCH3). 20.4 (CH3, CH2, C15, C17), 27.6 (CH2, C10, MS (ESI) m/z [M+Na]+ 4141.04).

1.6. Boc-D-Phenyl-methyl)-OH (8)

An aqueous solution of 1-MHOH (25 ml) was added to 0.7 g of 3 dissolved in THF (25 ml) and the reaction was monitored by TLC (ethyl acetate:hexane, 1:1). After completion of hydrolysis, the reaction mixture was acidified by an aqueous solution of 1-NHOH and the mixture was extracted with ethyl acetate (3 x 50 ml). The organic layers were combined, washed with H2O (2 x 20 ml), dried over Na2SO4, concentrated under reduced pressure, and lyophilized to give 0.6 g of 8 as a white solid. 1H NMR (400 MHz, CDCl3) δ 7.59 (d, 1 H, J 8.0 Hz, Ar-H), 7.29 (d, 1 H, J 8.0 Hz, Ar-H), 7.23 (dd, t, 1 H, J 7.4 Hz, Ar-H), 7.11 (dd, t, 1 H, J 7.4 Hz, Ar-H), 6.50 (s, 1 H, ind–2–H), 5.03 (br, s, 1 H, J 7.2 Hz, NHCCH3), 4.7 – 4.6 (m, 1 H, CH2), 3.74 (s, 3 H, NHCCH3), 3.38 – 3.25 (m, 2 H, CH2), 1.43 (s, 9 H, t-Bu). 2C NMR (75 MHz, CDCl3) assignments made using DEPT-135 δ: 176.5 (C-12), 155.5 (C-15), 137.1 (C-6a), 132.8 (C-9), 127.3 (CH-Ind), 121.8 (C-11), 119.4 (C-14a, C-14c), 119.2 (CH-Ind), 109.3 (C-14b). 104.9 (C-14c), 108.3 (C-14a), 104.9 (C-14b). 32.8 (CH3-NHCCH3). 20.4 (CH3, CH2, C15, C17), 27.6 (CH2, C10, MS (ESI) m/z [M+Na]+ 4141.04).
4.1.9. Boc-D-Trp(N-Methyl)-OH (11)

The method of synthesis was similar to that described for Boc-Tyr(Me)-OH. Boc-1-M Leu was added to 0.98 g of K2CO3 give 0.7 g (88%) of 11 as a white solid (mp: 147–148 °C, 74% NMR, CDCl3 δ: 7.59 (d, H-1), 7.46 (t, H-2), 7.35 (d, H-3), 7.31 (t, H-4), 4.16 (q, H-5), 3.67 (s, H-6), 3.54 (s, H-7), 3.22 (s, H-8), 1.37 (t, H-9), 1.20 (d, H-10, 15 NMR, 75 MHz, CDCl3 assignments made using DEPT-135): 1.690 (C-12), 1.558 (C-13), 1.644 (C-14), 1.284 (C-15, C-16, C-17), 2.37 (C-18), 2.80 (C-19, C-20), 15.3 (CH3-21), 58.0 (C-22), 60.8 (C-23). Accurate mass calculated for C15H21NO5: 250.14976. Found: MS (ESI) 359.1065.

4.1.10. Boc-D-Trp(N-Methyl)-OH (12)

The method of synthesis was similar to that described for Boc-Tyr(Me)-OH. 1-M Leu was added to 0.45 g of 7 to give 0.13 g (88%) of 12 as a white solid (mp: 103–104 °C, 74% NMR, CDCl3 δ: 7.59 (d, H-1), 7.46 (t, H-2), 7.35 (d, H-3), 7.31 (t, H-4), 4.16 (q, H-5), 3.67 (s, H-6), 3.54 (s, H-7), 3.22 (s, H-8), 1.37 (t, H-9), 1.20 (d, H-10, 15 NMR, 75 MHz, CDCl3 assignments made using DEPT-135): 1.690 (C-12), 1.558 (C-13), 1.644 (C-14), 1.284 (C-15, C-16, C-17), 2.37 (C-18), 2.80 (C-19, C-20), 15.3 (CH3-21), 58.0 (C-22), 60.8 (C-23). Accurate mass calculated for C15H21NO5: 250.14976. Found: MS (ESI) 359.1065.

4.2. Methionyl-H2N (13)

The crude product was purified using preparative RP-HPLC with the following linear gradient: 0–50% solution B from 0 to 5 min, 50–100% solution B from 5 to 45 min, and 100–100% solution B from 45 to 50 min, followed by a gradient of 50% A for 10 min. The product (13) was collected and lyophilized. RP-HPLC: tR 22.58 min. MS (APCI) m/z [M+H]+ 283.4. Accurate mass calculated for C10H17NO2S: 283.04446. Found: MS (APCI) 283.04458.

4.2.2. Ethyl-H2N (14)

The crude product was purified in 13 to give 14. RP-HPLC tR 2.18 min. MS (APCI) m/z [M+H]+ 81.9. Accurate mass calculated for C3H7NO: 81.04620.

4.2.3. Propyl-H2N (15)

The crude product was purified in 13 to give 15. RP-HPLC tR 2.58 min. MS (APCI) m/z [M+H]+ 93.4. Accurate mass calculated for C4H9NO: 93.04602.

4.2.5. Butyl-H2N (16)

The crude product was purified in 13 to give 16. RP-HPLC tR 2.74 min. 1H NMR (400 MHz, DMSO-d6) 8: 6.87 (s, H-1, 6 H), 6.82 (d, 2H, 6.8 Hz, 2 H), 6.13 (t, 2H, 6.8 Hz, 2 H), 7.04 (d, 2H, 6.8 Hz, 2 H), 3.78 (d, 2H, 6.8 Hz, 2 H), 2.98 (s, 2H, 6.8 Hz, 2 H), 2.59–2.49 (m, 4H, 6 H), 1.29 (t, 3H, 6 H), 0.92 (t, 3H, 6 H), 0.88 (s, 3H, 6 H). Accurate mass calculated for C10H21NO: 161.1647. Found: MS (APCI) 161.1650.

4.2.6. Pentyl-H2N (17)

The crude product was purified using preparative RP-HPLC with the following linear gradient: 0–50% solution B from 0 to 5

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min. 50–0.05 solution B from 5 to 45 min, and 0.05–100% solution B from 45 to 50 min, along with solution A. The peptide (27) was collected and lyophilized. RP-HPLC was performed on a 3.5 μm C18 column (Spherisorb ODS2, 150 × 4.6 mm, Waters) and eluted with a linear gradient of 0.1% aqueous TFA in water and 0.1% aqueous TFA in acetonitrile. The peptide was identified by LC-MS/MS analysis. The primary structure of the peptide was determined by mass spectrometry. The peptide was further purified by reverse-phase HPLC. The purity of the peptide was confirmed by analytical HPLC and LC-MS. The peptide was characterized by NMR spectroscopy and amino acid analysis. The peptide showed a high degree of purity and was suitable for biological studies.

5. Methodologies

5.1. Cell culture and growth conditions

RPMI-1640 (with glutamine and NaHCO3) was obtained from Sigma-Aldrich. Cells were cultured in 100 μl v/v FBS/RPMI-1640 (complete medium) and incubated at 37 °C in humidified atmosphere of 3% CO2. Both RPMI and DMEM cells are suspension cells and grow in aggregates. For the experimental part, cells were re-suspended in fresh complete medium.

5.2. Cell viability assays using resazurin dye

Dulbecco's PBS (without calcium chloride and magnesium chloride) and 10% fetal bovine serum were used as the basal medium. Cells were seeded into well plates at a seeding density of 1 × 10^5 cells/100 μl/well in triplicate. Cells were then treated with the compounds as described above. Cell viability was assessed after 48 h of incubation by a colorimetric assay using resazurin dye. Cells were incubated with resazurin dye for 4 h, and the absorbance at 570 nm was measured. The absorbance was recorded using a Tecan Spectra photometer. The absorbance values were used to calculate the percentage of cell viability.

5.3. Stability studies

Experiments were performed to determine the stability of the peptide in various biological samples. The stability of the peptide was assessed by incubating the peptide solution in different biological fluids (e.g., serum, plasma, and urine) at 37 °C for different time periods. The peptide was characterized by LC-MS/MS analysis before and after incubation to determine the degradation products and the remaining intact peptide. The data were analyzed using appropriate software to determine the half-life of the peptide in each sample.

6. Conclusion

The preparation of the peptide and its characterization by various methods provided substantial evidence for the synthesis of a novel peptide with potential therapeutic applications. The peptide was shown to be stable in various biological fluids, and its potential biological activity was further confirmed by in vitro cellular assays. The data presented in this study provide a foundation for further investigations into the potential therapeutic applications of the peptide.
enzymes activation was required and the aliquots were taken at 1, 2 and 3 h. The cofactors were added to initiate the study as previ- ous described. [27]. Briefly, stock concentrations of NADP+ (40 mm), UDPGA (20 mm), GSH (2 mm) and PAPS (2 mm) were prepared using Tris buffer (200 mm) (pH 7.4) containing 2 mm magnesium chloride. A fresh mixture of the four cofactors was prepared in volume ratio of 1:1:1:1, which was added to the sample containing the peptide and the 50 % lung fraction to result in final concentrations for the cofactors: NADP+ (1 mm), UDPGA (0.5 mm), GSH (0.05 mm) and PAPS (0.05 mm).

5.4. Assessment of apoptosis

5.4.1. Annexin V conjugate and flow cytometric analysis

The experiment was performed according to manufacturer’s instructions. Briefly, DM757 cells were seeded in 12-well plates at a seeding density of 1 × 10^5 cells/well in 1 ml of culture media. After 48 h incubation, 5 μl aliquots of 1:1 AODR mix (100 μg/ml in PBS of each dye) were added to each well and viewed under an inverted fluorescent microscope. Images were collected on an Olympus BX3 inverted microscope using a 10 x (0.3 Numerical objective lens and captured using an Ocular camera (Matsucam) through CellSens software (Olympus). Specific band pass filter sets for FITC and Texas Red were used to prevent bleed through from one channel to the next. Images were then processed and analyzed using Feli Image.

5.4.2. Annexin V conjugate and flow cytometric analysis

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jcbrech.2017.05.053.

References


